BIO-ANALYTICAL METHOD DEVELOPMENT AND VALIDATION OF GEFITINIB IN HUMAN PLASMA BY RP-HPLC AND DERIVATIVE SPECTROPHOTOMETRIC METHOD APPLIED TO THE DETERMINATION OF GEFITINIB IN BULK AND TABLET DOSGE FORM

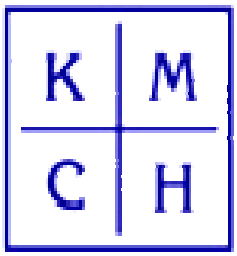
Dissertation Submitted to

The TamilNadu Dr. M.G.R. Medical University, Chennai.

In partial fulfillment for the award of the Degree of

MASTER OF PHARMACY
(Pharmaceutical Analysis)

APRIL-2012



DEPARTMENT OF PHARMACEUTICAL ANALYSIS

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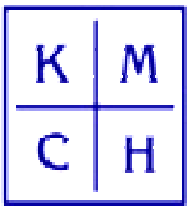

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CERTIFICATE

This is to certify that, the work embodied in the thesis entitled ***"BIO-ANALYTICAL METHOD DEVELOPMENT AND VALIDATION OF GEFITINIB IN HUMAN PLASMA BY RP-HPLC AND DERIVATIVE SPECTROPHOTOMETRIC METHOD APPLIED TO THE DETERMINATION OF GEFITINIB IN BULK AND TABLET DOSGE FORM"*** *is a bonafide research work carried out by Mr.* Ananth Vannemsetty ***(Reg. No: 26107221)****, Student in Master of Pharmacy, Department of Pharmaceutical Analysis, K.M.C.H. College of Pharmacy, Coimbatore, Tamilnadu, under the guidance of* ***Mr. I. Ponnilavarasan, Professor,*** *Dept of Pharmaceutical Analysis, K.M.C.H. College of Pharmacy during the academic year 2011-2012.*

Date:

Place: Coimbatore.

Signature,

Prof. Dr. A. Rajasekaran, M. Pharm, Ph.D.,
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I am fully satisfied with his performance and work with great pleasure. I forward this Dissertation work for evaluation.

Date:

Place: Coimbatore.

Signature,

Mr. I. Ponnilavarasan, M.Pharm, (Ph. D).,

Professor,

DECLARATION

I am here by stating that, to the best of my knowledge and belief, the project report entitled ***"BIO-ANALYTICAL METHOD DEVELOPMENT AND VALIDATION OF GEFITINIB IN HUMAN PLASMA BY RP-HPLC AND DERIVATIVE SPECTROPHOTOMETRIC METHOD APPLIED TO THE DETERMINATION OF GEFITINIB IN BULK AND TABLET DOSGE FORM"*** *being submitted for the partial fulfillment of Master of Pharmacy in Pharmaceutical Analysis for the academic year 2011-2012 of KMCH. College of Pharmacy affiliated to The Tamilnadu Dr. M.G.R. Medical University carried out under the guidance of* **Prof. I. Ponnilavarasan, M.Pharm.,(Ph.D).,** *at the Department of Pharmaceutical Analysis, KMCH College of Pharmacy, Coimbatore.*

I abide that all the data presented in this report will be treated with utmost confidentiality.

Signature,

Ananth Vannemsetty

Date:

Place: Coimbatore.

EVALUATION CERTIFICATE

This is to certify that, the work embodied in the thesis entitled ***"BIO-ANALYTICAL METHOD DEVELOPMENT AND VALIDATION OF GEFITINIB IN HUMAN PLASMA BY RP-HPLC AND DERIVATIVE SPECTROPHOTOMETRIC METHOD APPLIED TO THE DETERMINATION OF GEFITINIB IN BULK AND TABLET DOSGE FORM"*** *submitted by Mr.* Ananth Vannemsetty ***(Reg. No: 26107221),*** *to The Tamilnadu Dr. M.G.R. Medical University, Chennai, in partial fulfillment for the Degree of* ***Master of Pharmacy****, in* ***Pharmaceutical Analysis****, is a bonafide research work carried out by the candidate at K.M.C.H. College of Pharmacy, Coimbatore, Tamilnadu, the same was evaluated by us during academic year 2011-2012.*

Examination Center: KMCH College of Pharmacy, Coimbatore.

Date:

Internal Examiner **External Examiner**

Convener of Examinations

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My dissertation entitled ***"BIO-ANALYTICAL METHOD DEVELOPMENT AND VALIDATION OF GEFITINIB IN HUMAN PLASMA BY RP-HPLC AND DERIVATIVE SPECTROPHOTOMETRIC METHOD APPLIED TO THE DETERMINATION OF GEFITINIB IN BULK AND TABLET DOSGE FORM"*** *would not have been a feasible one without the grace of god almighty who gave me morale till the completion of my project.*

I am extremely thankful to my Academic Guide ***Mr. I. Ponnilavarasan, M. Pharm.,*** *Professor, Department of Pharmaceutical Analysis, KMCH. College of Pharmacy, for his constant insight, guidance, countless serenity, encouragement and painstaking efforts in my project work. I am indebted to his kindness and never failing co – operation.*

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Finally I would like to express my sincere thanks to all those people who directly or indirectly helped me to complete this work successfully.

Above all I dedicate myself before the unfailing presence of ***GOD*** *and constant love and encouragement given to me by my beloved* ***Father, Mother, Sister*** *and all of my family members who deserves the credit of success in whatever work I did.*

Yours sincerely,

Ananth Vannemsetty

ABBREVATIONS

HPLC	High Performance Liquid Chromatography
UV	Ultra violet
BA	Bioavailability
M.W.	Molecular weight
e.g.	Example
i.e.	That is
%	Percentage
PDA	Photo Diode Array
I.S	Internal Standard
ACN	Acetonitrile
MET	Methanol
RF	Response Factor
Mg	Milligram
mL	Milliliter
mM	Milli molar
µg	Microgram
w/w	Weight by weight

v/v	Volume by volume
µg/ml	Microgram per milliliter
ng /ml	Nanogram per milliliter
pH	Hydrogen ion concentration
^{0}C	Degree centigrade
T	Time
Abs.	Absorbance
Conc.	Concentration
Fig.	Figure
Tab.	Table
M.P.	Melting Point
AUC	Area under curve
CV	Coefficient of variance
RSD	Relative standard deviation
L/h	Liter per hour
L/kg	Liter per kilogram
Rpm	Rotation per minute
Rt	Retention time

INDEX

CHAPTER -1

INTRODUCTION

INTRODUCTION

Pharmaceutical Analysis[1-2] is the branch of chemistry involved in separating, identifying and determining the relative amounts of the components making up a sample of matter. It is mainly involved in the qualitative identification or detection of compounds and quantitative measurements of the substances present in bulk and pharmaceutical preparation.

Analytical instrumentation plays an important role in the production and evaluation of new products and in the protection of consumers and the environment. This instrumentation provides the lower detection limits required to assure safe foods, drugs, water and air.

TYPES

There are mainly two types of chemical analysis:

1. Qualitative (Identification)
2. Quantitative (Estimation)

1. Qualitative analysis is performed to establish composition of natural/synthetic substances. These tests are performed to indicate whether the substance or compound is present in the sample or not. Various qualitative tests are detection of evolved gas, formation of precipitates, limit tests, colour change reactions, melting point and boiling point test etc.

2. Quantitative analytical techniques are mainly used to quantify any compound or substance in the sample. These techniques are based on (a) the quantitative performance of suitable chemical reaction and either measuring the amount of reagent added to complete the reaction or measuring the amount of reaction product obtained. (b) The characteristic movement of a substance through a defined medium under controlled conditions. (c) Electrical measurement. (d) Measurement of some spectroscopic properties of the compound.

ULTRAVIOLET SPECTROSCOPY

Ultraviolet Spectroscopy is concerned with the study of absorption of UV radiation which ranges from 200 nm to 400 nm. Any molecule has 'n' or 'π' or 'σ' or a combination of these electrons. These bonding (σ and π) and nonbonding (n) electrons absorb the characteristic radiation and undergoes transition from ground state to excited state. By the characteristic absorption peaks, the natures of the electrons present and hence the molecule structure can be elucidated. UV spectroscopy works on Beer Lamberts law Principle.

Beer Lambert's law: It can be stated that as the intensity of beam of monochromatic light when passed through transparent medium decreases exponentially as the thickness and concentration of absorbing media increases arithmetically. Based on the readings we get on this principle Derivative Spectrophotometry depends.

Derivative Spectrophotometry [3 - 5]

Derivative Spectrophotometry [4] involves the conversion of a normal spectrum to its first, second or higher derivative spectrum and are shown in Fig: 1. In the context of Derivative Spectrophotometry, the normal absorption is referred to as fundamental, Zero th order or D_0 spectrum.

The First derivative spectrum (D^1) is a plot of the rate of change of absorbance with wavelength against wavelength, i.e. a plot of the slope of the fundamental spectrum against wavelength or a plot of $dA / d\lambda$ versus λ. The λmax is a wavelength of zero slope and gives $dA / d\lambda = 0$, i.e. a cross-over point, in the D^1 spectrum.

The Second derivative spectrum (D^2) is a plot of the curvature of the D^0 spectrum against wavelength or a plot of $d^2A / d\lambda^2$ versus λ. The maximum negative curvature at λ max in the D^0 spectrum gives a minimum in the D_2 spectrum.

In summary the First derivative spectrum of an absorption band is characterized by a maximum, a minimum, and a cross-over point at the λ max of the absorption band. The

Second derivative spectrum of an absorption band is characterized by two satellite maxima and an inverted band of which the minimum corresponds to the λ max of the fundamental band. The location of the end point of a potentiometric titration can often be accomplished more exactly from the First or Second derivative of the titration curve, than from the titration curve itself. Similarly, absorption observations will often yield more information from derivative plots than from the original absorption curve. This technique was used as long ago as 1955, but with the development of microcomputers which permit rapid generation of derivative curves, the method has acquired great impetus.

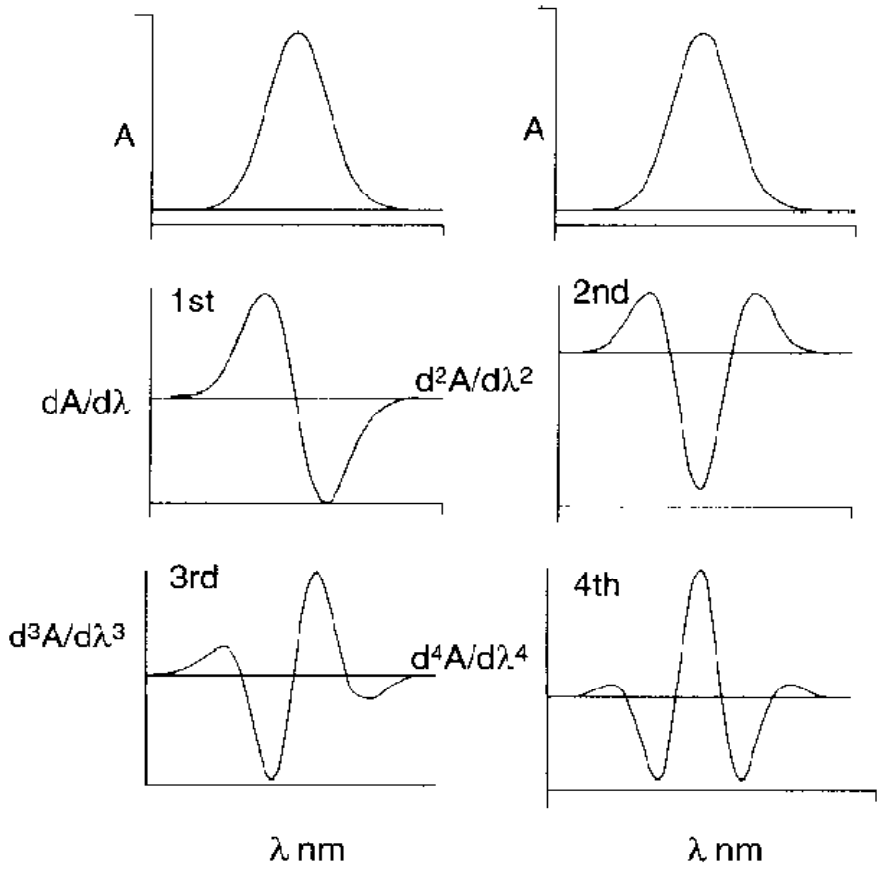


Fig: 1 Zero, First, Second, Third and Fourth order derivative curves [5]

If we consider an absorption band showing a normal (Gaussian) distribution, we find that the First and Third derivative plots are disperse functions that are unlike the original curve, but they can be used to fix accurately the wavelength of maximum absorption.

The Second and Fourth order derivatives have a central peak which is sharper than the original band but of the same height; its sign alternates with increasing order. It is clear that resolution is improved in the even-order spectra, and this offers the possibility of separating two absorption bands which may in fact merge in the Zero-order spectrum. Thus, a mixture of two substances gave a Zero-order spectrum showing no well-defined absorption bands, but the second-order spectrum deduced from this curve showed well resolved peaks. The influence of an impurity on the absorption spectrum of a substance can often be eliminated by considering derivative curves.

The Second-order plot of the mixture is identical with that of pure substance. When the interference spectrum can be described by an n^{th}-order polynomial, the interference is eliminated in the (n + 1) derivative.

For quantitative measurements peak heights (expressed in mm) are usually measured of the long-wave peak satellite of either the second- or fourth-order derivative curves, or for the short-wave peak satellite of the same curves. Derivative spectra can be recorded by means of a wavelength modulation device in which beams of radiation differing in wavelength by a small amount (1-2 nm) fall alternately on the sample cell and the difference between the two readings is recorded.

ADVANTAGES ON DERIVATIVE SPECTROPHOTOMETRY [3]

- ✓ A derivative spectrum shows better resolution of overlapping bands than the fundamental spectrum and may permit the accurate determination of the λ_{max} of the individual bands.
- ✓ It discriminates in favor of substances of narrow spectral bandwidth against broad bandwidth substances. This is because the derivative amplitude (D), i.e. the distance from a maximum to minimum, is inversely proportional to the fundamental spectral bandwidth (W) raised to the power (n) of the derivative order. Thus

$$D \alpha (1/W)^n$$

ROLE OF BIOANALYSIS IN PHARMACEUTICAL DRUG DEVELOPMENT [6]

Quantitative determination of drugs and their metabolites in biological fluids is termed as bioanalysis. This technique is used very early in the drug development process to provide support to drug discovery programs on the metabolic fate and pharmacokinetics of chemicals in living cells and in humans.

Bioanalytical methods play a major role in estimating the drugs, interferences, metabolites from various matrices such as pure drug, dosage form, intermediates and biological fluids. Drug assay technology is now sufficiently advanced for it to be possible to measure the plasma concentration of majority of drugs used in clinical practices. They are useful to measure plasma concentration of drugs to confirm adequate dosage, to identify signs of possible drug toxicity, the response of patients to drug therapy and drug interactions.

When a drug is administered orally it passes through the GIT and enters the systemic circulation undergoes metabolism, finally it is excreted as such or in the form of its metabolites. The studies on biological fluids is very challenging and time consuming , but these studies are necessary and utmost important because biological fluids like blood, urine, cerebrospinal fluid and milk etc containing a relative quantity of drug and their metabolites can be known. Bio-analytical method which gives accurate and reproducible results has been increased significantly now a day.

Therapeutic efficacy of the particular drug can be known by bioanalysis. In pharma field bioanalysis plays a significant role .Bioanalysis involves the following steps.

- Selection and collection of biological fluid.
- Preparation of sample –Analyte extraction from biological matrix.
- Analyte detection done by various methods.

In bioanalytical drug analysis, common matrices are venous blood and plasma. Venous blood, with anticoagulant, is after centrifugation separated into plasma and blood cells. Centrifuged blood consists of 55% plasma and 45% blood cells. The plasma matrix is composed of some 90% water with remaining 10% being mostly plasma proteins. Sampling of biological matrix is the first step in the analytical chain, and these samples must regularly

be stored in a cooler or freezer before assay. When the samples have arrived at the laboratory, they need to be prepared for analysis. The aim is to improve the assay by removing interferences in the matrix, and often also concentrating the analyte. The need of sample preparation is determined by the complexity of the samples and by the application of the analytical method.

Detection of drug or its metabolite in biological media is usually complicated by the matrix. Because of this, various types of cleanup procedures involved i.e. solvent extraction and chromatography are employed to effectively separate drug components form endogenous biological materials. The sensitivity and selectivity of the assay method was limited by the efficiency of the clean up methodology [7].

If the blood is allowed to clot and is then centrifuged, about 30 to 50% of the original volume is collected as serum (upper level). Thus, plasma generally is preferred because of its greater yield from blood. Blood, serum or plasma samples can be utilized for bioanalytical studies and may require protein denaturation steps before further processes.

If plasma or serum is used for the analytical procedure, the fresh whole blood should be centrifuged immediately at 5000 rpm for approximately 5 to 10 min, and the supernatant should be transferred by means of a suitable device, such as pasture pipette, to a clean container of appropriate size of storage.

DIFFERENT TERMINOLOGY USED IN BIOANALYTICAL DEPARTMENT

Accuracy: The degree of closeness of the determined value to the nominal or known true value under prescribed conditions. This is sometimes termed trueness.

Analyte: A specified chemical moiety being measured or which can be intact drug, biomolecule or its derivative, metabolite and/or degradation product in a biological matrix.

Analytical run (or batch): A complete set of analytical and study samples with appropriate number of standards and QCs for their validation. Several runs (or batches) may be completed in one day, or one run (or batch) may take several days to complete.

Biological matrix: A discrete material of biological origin that can be sampled and processed in a reproducible manner. Examples are blood, serum, plasma, urine, feces, saliva, sputum and various discrete tissues.

Calibration Standard: A biological matrix to which a known amount of analyte has been added or *spiked*. Calibration standards are used to construct calibration curves from which the concentrations of analytes in QCs and in unknown study samples are determined.

Internal standard: Test compound(s) (e.g. structurally similar analog, stable labeled compound) added to both calibration standards and samples at known and constant concentration to facilitate quantification of the target analyte(s).

Matrix effect: The direct or indirect alteration or interference in response due to the presence of unintended analytes (for analysis) or other interfering substances in the sample.

Method: A comprehensive description of all procedures used in sample analysis

Precision: The closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogenous sample under the prescribed conditions.

Processed: The final extract (prior to instrumental analysis) of the sample that has been subjected to various manipulations (e.g., extraction, dilution, concentration).

Recovery: The extraction efficiency of an analytical process, reported as a percentage of the known amount of an analyte carried through the sample extraction and processing steps of the method.

Reproducibility: The precision between two laboratories. It also represents precision of the method under the same operating conditions over a short period of time.

Sample: A generic term encompassing controls, blanks, unknowns and processed samples as described below.

Blank: A sample of a biological matrix to which no analytes have been added that is used to assess the specificity of the bioanalytical method.

Quality control sample (QC): A spiked sample used to monitor the performance of a Bioanalytical method and to assess the integrity and validity of the results of the unknown samples analyzed in an individual batch.

Unknown: A biological sample that is the subject of the analysis.

Selectivity: The ability of the Bioanalytical method to measure and differentiate the analytes in the presence of the components that may be expected to be present. These could include metabolites, Impurities, degradants or matrix components.

System suitability: Determination of instrument performance (e.g., sensitivity and chromatographic retention) by analysis of a reference standard prior to running the analytical batch.

VALIDATION:

Full validation: Establishment of all validation parameters to apply to sample analysis for the Bioanalytical method for each analyte.

Partial validation: Modification of validated bioanalytical methods that do not necessarily call for full validation.

Cross-validation: Comparison validation parameters of two bioanalytical methods.

STORAGE REQUIREMENTS FOR BIOLOGICAL SAMPLES [8]

In order to avoid decomposition or other potential chemical changes in the drugs to be analyzed, biological samples should be frozen immediately upon collection and thawed before analysis. When drugs are susceptible to plasma esterase, the addition of esterase inhibitors, such as sodium fluoride was immediately added after collection helps to prevent decomposition.

When collecting and storing biological samples, the analyte should be contaminated with storage vessels. For example, plastic –ware frequently contains high boiling liquid bis (2-ethylhexyl) phthalate; similarly, the plunger-plugs of vacutainers are known to contain tri-butoxyethyl phosphate, which can be interfering certain drug analysis.

EXTRACTION PROCEDURE FOR BIOLOGICAL SAMPLE

Sample preparation is a technique used to clean up a sample before analysis and/or to concentrate a sample to improve its detection. When samples are biological fluids such as plasma, serum or urine, this technique is described as bioanalytical sample preparation.

Objectives of Bio-analytical sample preparation:-

1. Removal of unwanted matrix components (primarily protein) that would interfere with analyte determination.
2. Concentration of analyte to meet the detection limits of the analytical instrument.
3. Exchange of the solvent or solution in which the analyte resides so that it is compatible with mobile phase for injection into a Chromatographic system.
4. Dilution to reduce solvent strength or avoid solvent incompatibility.
5. Stabilization of analyte to avoid hydrolytic or enzymatic degradation.

Blood, serum and plasma contain approximately 10,000 different proteins with a total concentration of 6–8 g/dL, and urine contains 50–100 mg of protein/dL. Plasma also contains approximately 3 mg/mL of sodium, 0.2 mg/mL of potassium, 15–38.5 mg/dL of urea, and many other organic and inorganic compounds. With such complex mixtures and the dynamic ranges of proteins, fatty acids, lipids and salts, it is clear that it is important to remove these components from the analysis, as these factors could confound the analysis and flood the detector with irrelevant ions.

GENERAL TECHNIQUES FOR SAMPLE PREPARATION [9]:-

Many different sample preparation techniques are available for choosing a method to perform bioanalytical sample preparation. These techniques vary in many regards, such as simplicity, time requirement (in terms of speed and hands-on analyst time), ease of automation, extraction chemistry expertise, concentration factor and selectivity of the final extract.

Typical choices of sample preparation techniques useful in bioanalysis

- Dilution followed by injection
- Solid Phase extraction

- Protein precipitation
- Filtration
- Liquid-liquid extraction
- Restricted access media

1.1.1. Solid phase extraction

Solid phase extraction (SPE) is one of the most common bio-analytical extraction methods in publication literature, a search of journal articles using the science direct search engine for the term "SPE" yielded 151,017 hits, with 4,657 hits for the year 2010 alone. SPE involves a solid-liquid phase separation of the analytes from the biological sample, by the selective transfer between a liquid and solid state [9]. The analyte is physically separated from the biological matrix by the differential interacting with a solid phase sorbent material. These sorbents, packed primarily into either disposable cartridges or discs, can be polar, non-polar or ionic depending on the experimental requirements. Solid phase extraction is based on four distinct protocol steps as illustrated in Figure 1-1.

Step 1: **Sorbent Conditioning**

Prior to the addition of the sample conditioning and packing of the cartridge is done. In order to remove the impurities a conditioning strong solvent like methanol is passed through cartridge which allows sorbent to be solvated.

Step 2: **Sample Loading**

In a weak solvent the sample is dissolved and passed through the cartridge. Sample is applied with a syringe or pipette or pumped into cartridge .This weak solvent helps in retention of strong analyte.

Step 3: **Washing**

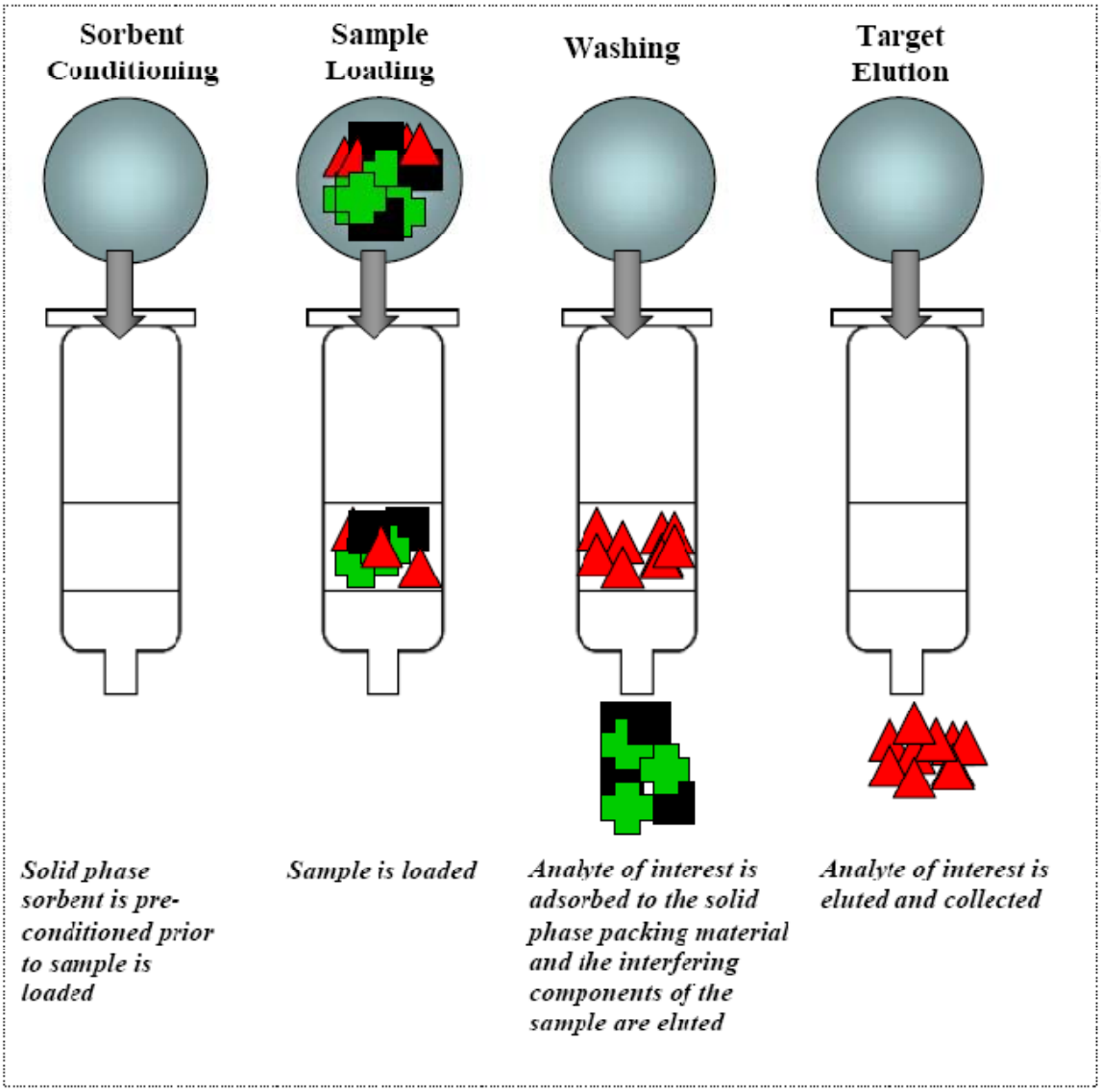
In order, to remove the interferences and impurities the cartridge is washed with water or buffer.

Step 4: **Analyte Elution**

Strong elution solvent is passed through the cartridge and it gives 100% collection of analyte from this method.

The availability of commercially available solid phase extraction material, cartridges or sorbents, has led to uniformity in analysis and makes method replication and transfer from one lab to another more straightforward. SPE is also frequently used in on-line extraction. Automation of sample clean-up is advantageous as it decreases the high level of manual handling and error associated with manual extraction procedures. Though on-line SPE is faster and allows for increased through-put injections and offers high recoveries, extensive method development is required to optimize many experimental variables.

Figure 1-1: Schematic of solid phase extraction procedure



1.2. Protein precipitation

Protein precipitation is based on the interaction between the precipitation reagent and protein groups. Soluble proteins generally have a hydrophobic core surrounded by a hydrophilic surface including ionic groups that are not involved in intra-molecular binding. Organic solvents interfere with the intra-molecular hydrophobic interactions of proteins. [10]

The addition of a volume of solvent (frequently acetonitrile) to the serum causes the proteins of the serum to precipitate and leaves the analyte of interest in the solvent, which can either be injected directly or dried down and reconstituted in a smaller volume to concentration before injection. While this is the fastest and simplest method for sample preparation, it is the most likely to cause ion suppression issues, especially in ESI, where the co-elution of endogenous compounds such as lipids, phospholipids and fatty acids affect the ESI droplet desolvation process. [11]

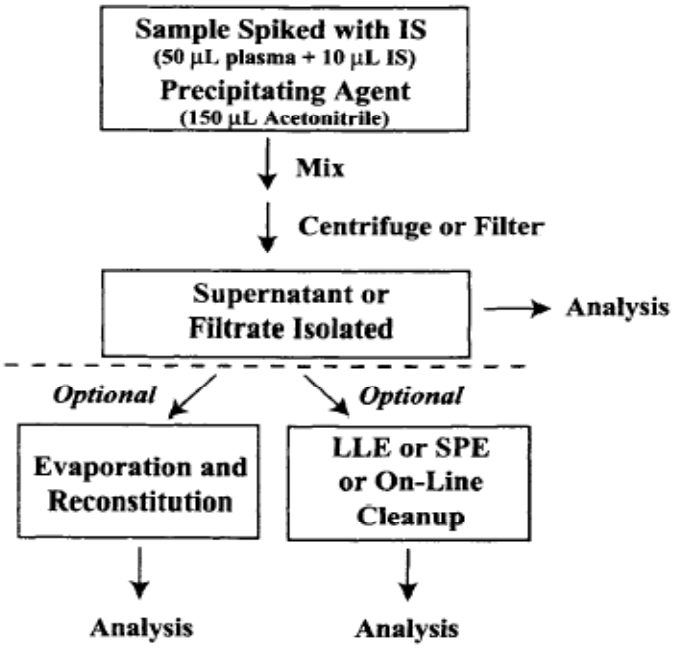
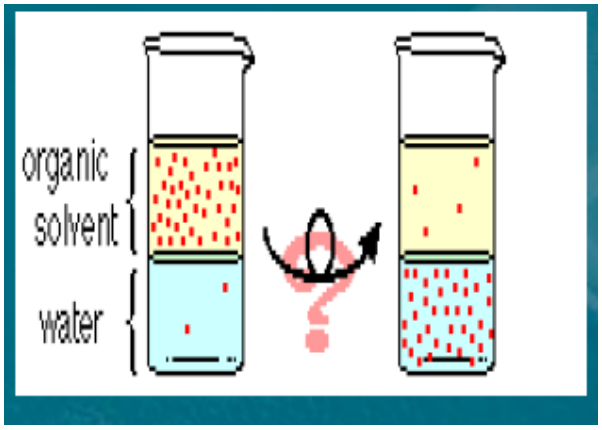


Figure 1-2 :- Schematic diagram of Protein precipitation technique.

1.1.3. Liquid-Liquid Extraction [12]

Liquid-liquid extraction (LLE) is the simplest form of the extraction and purification of analytes from liquid samples. The basic principle is the tendency of an analyte to prefer one solvent over another immiscible solvent. The often-quoted partitioning coefficient of a species is Log P. This is defined as the ratio of the concentration in octanol divided by the concentration in water. The Log-P values of a vast number of species have been calculated, and there are computer programs that will calculate Log P and Log D simply from the chemical structure of the species. The use of high-purity solvents can make this technique expensive, especially if large volumes are required and then there is the disposal of these solvents. Carrying out LLE requires the use of two immiscible liquids and soluble samples. It is very useful for separating analytes from interferences by partitioning the sample between these two immiscible liquids or phases. Typically, with LLE one phase will be aqueous (often the denser or heavier phase) and the second phase is an organic solvent (usually the lighter phase). Hydrophilic compounds prefer the polar aqueous phase, whereas hydrophobic compounds will prefer the organic solvent. Analytes extracted into the organic phase are recovered relatively easily by evaporation of the solvent, whereas analytes extracted into the aqueous phase are often amenable to direct analysis by high-performance liquid chromatography (HPLC). Extraction efficiency can be improved by solvent selection, solvent volume, pH, and by using the salting-out effect.



Positive –

- ✓ Known and standard technique.
- ✓ Relatively simple to do as a routine.
- ✓ Trend to micro LLE.
- ✓ Low LOD's are possible.

Negative –

- ✓ Moderate selective (limited in solvent selections).
- ✓ Examine and use pH / ionic strength / temperature to get a selective extraction process.
- ✓ Multiple extractions needed to get the recovery.
- ✓ Often evaporation steps needed.
- ✓ Emulsions formed cause recovery loss.
- ✓ Difficult to automate, semi-automated steps.
- ✓ As routine method labour intensive.

Analytical methods for quantitative determination of drugs in biological fluids:

There are different types of methods for quantitative determination of drugs in biological fluids. According to biological fluid and drug to be quantitated these methods are selected. They are:

a) Methods based on immunoassay procedure
   - ✓ Radioimmunoassay (RIA).
   - ✓ Enzyme-multiplied immunoassay technique.
   - ✓ Enzyme-linked immunosorbent assay (ELISA).

b) Microbiological methods.

c) Capillary electrophoresis.

d) Chromatographic methods.
   - ✓ Gas chromatography (GC).
   - ✓ High performance liquid chromatography (HPLC).
   - ✓ Liquid chromatography-mass spectroscopy (LC-MS).
   - ✓ Gas chromatography –mass spectroscopy (GC-MS).

Chromatographic methods are mostly used and important for the estimation of drugs in biological samples. Chromatography derived from "*chroma"* means "color" and "*graphein"* means "to write". Separation of required analyte from the compounds by using the mobile phase and stationary phase is known as chromatography. Advanced methods for separation of samples from biological fluids are HPLC and their hyphenated methods like LC-MS; GC-MS.

Drugs estimation in biological samples by HPLC [13]

High-performance liquid chromatography [HPLC] is now one of the most powerful tools in analytical chemistry. It has the ability to separate, identify and quantitate the compounds that are present in any sample that can be dissolved in a liquid. Today, compounds in trace concentrations as low as parts per trillion (ppt) may easily be identified. HPLC can be and has been applied to just above any sample, such as

pharmaceuticals, food, nutraceuticals, cosmetics, environmental matrices, forensic samples and industrial chemicals.

HPLC is historically divided into two different sub classes based on the polarity of the mobile and stationary phases.

1. Normal phase high performance liquid chromatography.
2. Reverse phase high performance liquid chromatography.

Normal phase high performance liquid chromatography:

Techniques in which the stationary phase is more polar than the mobile phase is call normal phase high performance liquid chromatography.

✓ Stationary Phase –Polar nature

e.g.: Sio_2, Al_2O_3.

✓ Mobile Phase – Non-polar nature

e.g.: Heptane, hexane, cyclohexane, CHCL3, CH3OH

Mechanism:

- Polar compounds travels slower and eluted slowly due to higher affinity b/w solute and stationary phase.
- No polar compound travels faster and eluted first due to lower affinity b/w solute and stationary phase.
- This technique is not widely used in pharmaceutical separation.

Reverse phase high performance liquid chromatography:

Techniques in which the mobile phase is more polar than the stationary phase is called reverse phase high performance liquid chromatography.

✓ Stationary phase – Non-polar nature.

e.g.: n-octadecyl, n-octyl, ethyl, phenyl diol, hydrophobic polymers.

✓ Mobile Phase – polar nature.

e.g.: Methanol or Acetonitrile/water or buffer sometimes with additives of THF or dioxane.

Mechanism:

- A polar compound travels faster and eluted first due to lesser affinity b/w solute and stationary phase.
- Non polar compounds travels slower and eluted slowly due to higher affinity b/w solute and stationary phase.

Mobile phases used in HPLC

In HPLC a broad variety of mobile phases can be used. According to the mode of HPLC mobile phase can be selected like polar nature or non-polar. Mobile phase selection depends on many factors like purity, sample solubility, chemical inertness, detector compatibility and low viscosity. Mostly organic solvents like acetonitrile, methanol, ethanol, formic acid etc are used. Sometimes these solvents are mixed with water and also can be used. These mobile phases should be selected according to the properties of drug to be estimated in HPLC, so its selection should be done carefully. Based on the mobile phase only the drug elutes and chromatogram of that drug comes properly.

LISTS OF STEPS NEEDED BEFORE ANY RUN BY HPLC [14]:

- ✓ Filter the solvents with membranes with cut off 0.22-0.45m.
- ✓ Precipitates and colloids can be distinguished by using clean and transparent reservoirs.
- ✓ Make sure that the solvents will be easily mixed with the previous solvents in the same inlets. For example methanol or water should not be placed instead of hexane directly, or any organic solvent should not be placed directly instead of a buffer reservoir.
- ✓ Degas the solvents and remove by cleansing all the tubing that lead to the pump.
- ✓ Connect the column according to the flow direction indicated on it (do not connect directly to the detector).
- ✓ At a low flow rate stream the appropriate the solvents through the column (0.1-0.5 ml/min) or reach the composition gradually using the appropriate gradient.

- ✓ Wait for a stable baseline and select an appropriate wavelength in the detector.
- ✓ Prepare the set of methods in the workstation: Instrument method for the control on the system, processing method for the data processing and the report method for the report of final results.
- ✓ A blank run should be performed to test the system and verify that it is clean from interferences when the methods are ready.

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY:

- ✓ In 1903, Mikhail Tswett discovered Chromatography technique. It serves as a means of resolution of mixtures. The name suggests ***chroma*** meaning "colour" and ***graphein*** meaning "write".
- ✓ HPLC is one of the types of Chromatography. In modern pharmaceutical industries, HPLC is the major and integral analytical tool applied in all stages of drug discovery, development, and production. Effective and fast method development is of paramount importance throughout this drug development life cycle. This requires a thorough understanding of HPLC principles and theory which lay a solid foundation for appreciating the many variables that are optimized during fast and effective HPLC method development and optimization.

Principle:

- ✓ The principle of separation is normal phase mode and reverse phase mode is adsorption. When mixtures of components are introduced in to a HPLC column, they travel according to their relative affinities towards the stationary phase. The component which has more affinity towards the adsorbent travels slower.
- ✓ The component which has less affinity towards the stationary phase travels faster. Since no two components have the same affinity towards the stationary phase, the components are separated.
- ✓ Most of the drugs in multicomponent dosage forms can be analyzed by HPLC method because of the several advantages like rapidity, specificity, accuracy, precision and ease of automation in this method. HPLC method eliminates tedious extraction and isolation procedures.

Minimum requirement for HPLC: [15]

A).Temperature:

Room temperature is the first choice. Elevated temperatures are sometimes used to reduce column pressure are enhancing selectivity. Typically, temperatures in excess of $60^{o}C$ are not used.

B).Retention time mechanism:

In general, HPLC is a dynamic adsorption process. Analyte molecules, while moving through the porous packing bead, tend to interact with the surface adsorption sites. Depending on the HPLC mode, the different types of the adsorption forces may be included in the retention process:

- Hydrophobic (non-specific) interactions are the main ones in Reversed-Phase Separations.
- Dipole-dipole (polar) interactions are dominated in normal phase mode.
- Ionic interactions are responsible for the retention in ion-exchange Chromatography. All these interactions are competitive. Analyte molecules are competing with the eluent molecules for the adsorption sites. So, the stronger analyte molecules interact with the surface and the weaker the eluent interaction, the longer analyte will be retained on the surface.

Significance of drug analysis in body fluids:

Bioanalytical studies have many important applications in pharmacy field. They are mainly:

- Bioequivalence studies.
- Forensic and toxicological studies.
- Drug –drug interaction and Bioavailability.
- Therapeutic drug monitoring (TDM).
- Adverse effect.
- Over dose.
- Drug abuse in sports.
- Pharmacokinetic studies and In clinical trials.

Quantitative Analysis by HPLC [16]:-

Three methods are generally used for quantitative analysis in HPLC. They are the external standard method, the internal standard method and standard addition method

- ***External standard method***

The external standard method involves the use of single standard or up to three standard solutions. The peak area or the height of the sample and the standard used are compared directly or the slope of calibration curved based on standards that contain known concentration of the compound of interest.

- ***Internal standard method***

A widely used technique of quantitation involves the addition of an internal standard to compensate for various analytical errors. In this approach, a known compound of a fix concentration is added to the known amount of sample to give separate peak in the chromatograms, to compensate for the losses of the compound of interest during sample pretreatment steps. Any loss of the compound of interest will be accompanied by the loss of an equivalent fraction of internal standard. The accuracy of this approach obviously depends on the structural equivalence of the compounds of interest and the internal standard.

The requirement for an internal standard must

- ✓ Give a completely resolved peak with no interferences
- ✓ Elute close to the compound of interest
- ✓ Behave equivalent to the compound of interest for analysis like pretreatment, derivative formation, etc.
- ✓ Be added at a concentration that will produce a peak area or peak height ratio of about unity with the compound of interest.
- ✓ Not be present in original sample
- ✓ Be stable, unreactive with the sample components, column packing and the mobile phase and
- ✓ Be commercially available in high purity

The internal standard should be added to the sample prior to sample preparation procedure and homogenized with it .Response factor is used to determine the concentration of a sample component in the original sample. The response factor (Rf) is the ratio of peak area of sample component (A_x) and the internal standard (A_{IS}) obtained by injecting the sample quantity.

❖ ***Standard addition method:***

In this method for the sample solution known amount of standard is added. By plotting calibration curve graphically the quantitative analysis is carried out. This method is used for sample which contains which contains very small quantity of element to be analyzed. In trace analysis also this method is used. By adding the standard to sample solution the peak area is increased and concentration of sample will be computed by interpretation.

Validation:

Validation is a process of stabling documented evidence, where high degree of assurance can be provided, that a specific activity will consistently produce a desired result or product meeting its predetermined specifications and quality characteristics [17].

Validation is very important for a method. This has to be compulsory done after the method development. In order, to prove that process is consistently doing

1. What it is supposed to do.

2. To determine the process variable and acceptable limits for those variables and to set up appropriate in- process control.

3. To assure the quality and to see that product is prepared in the reasonable amount of time.

Although there are various stages in the development and validation of an analytical procedure, the validation of the analytical method can be envisaged to be to consist of two distinct phases:

a) The ***pre-study phase*** which comes before the actual start of the study and involves the validation of the method on biological matrix human plasma samples and spiked plasma samples.

b) The ***study phase*** in which the validated bioanalytical method is applied to the actual analysis of the samples from bioavailability and bioequivalence studies mainly to confirm the stability, accuracy and precision.

Bioanalytical method validation (BMV) [18]

For quantitative determination of drugs and their metabolites in the in biological matrix bioanalytical method validation (BMV) is done and this gives information about various things like pharmacokinetic, bioequivalence, bioavailability, toxicology studies. For regulatory filings these studies will support. It is necessary to emphasize well-characterized and fully validated analytical methods to produce reliable results that can be satisfactorily interpreted. For each analytical method has its own characteristics which may vary from analyte to analyte in these instances. So, specific validation criteria is essential, to be developed for each analyte. Most of the procedures, principles, and requirements for quantitative bioanalytical method validation are common to all types of analytical methods. In bioanalytical method validation two phases are present. They are:

(1) The bioanalytical method development phase in which the assay is defined and validated.

(2) The application to actual analysis of samples from pharmacokinetic, bioavailability, bioequivalence, and drug interaction studies.

In bioanalytical method validation (BMV) validation is done by three different levels/types of validation are done. They are Full validation, Partial validation, Cross validation.

Full validation:

It is done when developing bioanalytical method for first time for a new drug. Full validation is performed when an existing assay for quantification was added by metabolites.

Partial validation:

These are modifications to bioanalytical methods for which Full validation is not necessary. Modifications done for bioanalytical methods such as, small changes like change in species with matrix (from rat plasma to mouse plasma),change in matrix with in a species (from human urine to human plasma) change in laboratories or analysts, instruments, change in sampling process procedures, change in analytical method like changing detector.

Cross validation:

In this two bioanalytical methods are compared. The "reference" method which is original one is compared with the revised one "comparator". This is done where two bioanalytical methods are compared and from that same data is prepared for study. This is done in two ways. Spiked matrix samples and subjected samples validation done at sane site or done at different sites i.e. different laboratories or by using different techniques in same laboratory.

FDA guidance on bioanalytical method validation [19]:

For bioanalytical method validation FDA had given some guidelines. These guidelines are given the validation performed was to be accurate. They are:

- Analyte stability in biological matrix at intended storage and operating conditions should be kept.
- Standard curve for matrix-based one should contain minimum 5 standards without including blank and it should cover the entire range of concentrations expected.
- Essential parameters that are performed for acceptability of bioanalytical method are precision, accuracy, selectivity, sensitivity and reproducibility.
- For lowest concentration on the standard curve the lower limit of quantification can be used and that is not confused with limit of detection.
- During the Full validation of a bioanalytical method the below parameters should be defined.

Validation parameters [20]:

Different Types of Validation characteristics:

- Precision
  - Repeatability
  - Intermediate Precision
  - Reproducibility
- Accuracy
- Specificity Selectivity
- Linearity Range

- Limit of Detection (LOD)
- Limit of Quantification (LOQ)
- Robustness
- Ruggedness.
- System Suitability

1) Precision:

When the procedure is applied repeatedly to multiple samplings of single homogenous sample under prescribed conditions then precision, is a closeness of individual measurements of the analyte. It is done at three levels such as repeatability, intermediate precision, and reproducibility.

Repeatability: It expresses precision under same operating conditions i.e. with in the laboratory same analyst using same equipment over a short period of time.

Intermediate precision: It is the precision under different laboratory conditions i.e. varying only in different analyst, on different days, or using different equipments within the same laboratory.

Reproducibility: It is the precision between different laboratories and is often determined in method transfer experiments

Acceptance Criteria:

- ✓ Percentage Relative deviation (%RSD) NMT 1 % (Instrument precision)
- ✓ (%RSD) NMT -2% (Intra- assay precision)

2) Accuracy:

It is the closeness of mean tests results obtained by the method to true concentration of analyte. It is also named as trueness. Accuracy is determined by replicate analysis of samples containing known amounts of the analyte. Most commonly used method for determination of accuracy is recovery studies. The usual range is being 10% above or below the expected range of claim. The % recovery was calculated using the formula,

$$\%\,\mathrm{Recovery} = \frac{(a+b)-a}{bX100}$$

Where,

a – Amount of drug present in sample

b – Amount of standard added to the sample

Acceptance Criteria:

- In assay method, mean recovery will be 100% ± 2% at each concentration between the ranges of 80-120% of the target concentration.
- In impurity method, mean recovery will be 0.1% absolute of the theoretical concentration or 10% relative, whichever is greater for impurities between the ranges of 0.1-2.5 % (V/W).

3) Limit of detection (LOD):

The limit of detection is the lowest concentration of analyte in the sample which can be detected but not quantified under given experimental conditions. The lowest concentration which can be distinguished from the background noise with a certain degree of confidence is defined as limit of detection. Prepare the blank solution as per test method and inject six times into the chromatographic system. Similarly prepare the linearity solution staring from lowest possible concentration of analyte to 150 % (or as per protocol) of target concentration and establish the linearity curve.

The detection limit (DL) may be expressed as:

$$\mathrm{LOD} = \frac{3.3 \times \text{Standard deviation of the response of the blank } (\sigma)}{\text{Slope}}$$

The slope shall be estimated from the calibration curve of the analyte.

4) Limit of quantification (LOQ):

It is also the lowest concentration of analyte in the sample but quantitatively determined with suitable accuracy and precision.

In calibration curve it is the lowest concentration point. It is determined by accuracy by the presence of background signal and by precision i.e. reproducibility of analyte in the method.

Acceptance Criteria:

- In Pharmaceutical application, the LOQ is typically set at minimum 0.05% for active pharmaceutical ingredients.
- LOQ defined as the lowest concentration providing a RSD of 5%.
- LOQ should be at least 10% of the minimum effective concentration for clinical applications.

5) Specificity:

A method is said to be specific when it produces proper response only for a single analyte. It can be demonstrated by performing Placebo / blank interference and forced degradation studies. If the expected impurities or related substances are available, then they should be analyzed along with the analyte or sample to check the system suitability, retention factor, tailing factor and resolution etc. In this peak purity studies are done for specificity.

6) Linearity:

As per ICH definition "the ability to obtain test results which are directly proportional to the concentration of an analyte within given range is known as linearity of an analytical procedure". By using correlation coefficient this can be tested. Using correlation coefficient is a benefit as it is a relationship between concentration and response data. In this data is analyzed by linear least square regression co-efficient and b of the linear equation,

$$Y = a.X + b$$

By the above equation regression **r** value can be known. For the method to be linear the **r** value should be close to1. Where **Y** is the measured output signal, **X** is the concentration of sample, **a** is the slope, **b** is the intercept.

Acceptance criteria:

Coefficient of correlation should be NLT 0.99.

7) Robustness:

It is the measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage.

8) Ruggedness:

Ruggedness according to the USP is "the degree of reproducibility of test results obtained by the analysis of the same samples under a variety of normal test conditions, such as different labs, different analysts, and different lots of reagents. The following are the typical method parameters need to test during method validation:

- Analyst-to-Analyst variability.
- Column-to-Column variability.
- On different days.
- In different laboratories.

APPLICATION OF A VALIDATED BIOANALYTICAL METHOD TO ROUTINE DRUG ANALYSIS [21]

Many of the above principles under method establishment and validation are relevant to within study validation. This section will emphasize the validation parameters that should be evaluated during routine application of a validated bioanalytical method to a particular study. Following a successful validation which meets an *a prior* set acceptance criteria for accuracy and precision, examination of biological samples can be done by single determination without a need for duplicate or replicate analysis. The need for duplicate analysis may arise for special cases. For example, in the case of a difficult procedure for labile analyte (s), when the precision and accuracy tolerances are difficult to achieve, duplicates analyses may be essential.

A matrix-based standard curve should be generated for each analytical batch for each analyte and should be used in calculating the concentration of analyte in the unknown samples assayed with that run. It is important to use a matrix based standard curve that will cover the entire range of concentrations in the unknown samples. Estimation of unknowns by extrapolations of standard curve below the LLOQ or above the ULOQ is not recommended. Instead, it is recommended that the standard curve be re-determined or samples be reassayed after dilution with the matrix.

- ✓ A matrix-based standard curve should consist of a minimum of five to eight standard points, excluding blanks (either single or replicate), covering the entire range.
- ✓ Response Function: Typically, the same curve fitting, weighting and goodness of fit determined during prestudy validation should be utilized for the standard curve within study. Response function is determined by appropriate statistical tests based on the actual standard points during each run in the validation.
- ✓ The QC samples should be used to accept or reject the run. These QC samples are matrix spiked with analyte.
- ✓ System suitability: Based on the analyte and technique, a specific standard operating procedure (or sample) can be identified to assure the optimum operation of the system employed.
- ✓ Any required sample dilutions must utilize like matrix (e.g. human to human) obviating the need to incorporate actual within-study dilution matrix QC samples.

CHAPTER -2

LITERATURE REVIEW

LITERATURE REVIEW

Pranay wal et al [22], 2010 : had reported the major challenges facing the pharmaceutical industry today is finding new ways to increase productivity, decrease costs whilst still ultimately developing new therapies that enhance human health. One of the major challenges facing the pharmaceutical industry today is finding new ways to increase productivity, decrease costs whilst still ultimately developing new therapies that enhance human health. To help address these challenges the utilization of analytical technologies and high-throughput automated platforms has been employed; in order to perform more experiments in a shorter time frame with increased data quality. During the last decade, quantification of low molecular weight molecules using liquid chromatography–tandem mass spectrometry in biological fluids has become a common procedure in many preclinical and clinical laboratories. This overview highlights a number of issues involving "small molecule drugs", bioanalytical liquid chromatography–tandem mass spectrometry, which are frequently encountered during assay development. Since plasma is one of the most widely adopted biological fluid in drug discovery and development, the focus of this discussion will be limited to plasma analysis. Bioanalytical method development largely depends on the experience and the preference of the developer. Mathematical models could help in selecting the proper conditions to develop a selective and robust method, using liquid chromatography, liquid–liquid extraction, solid phase extraction and protein precipitation Special attention has been paid to matrix effects, the most important issues in bioanalysis and possible solutions to handle these issues are discussed. By proper use of the proposed models a more structured method development is accomplished, resulting in a description of the method that could be used for future use to control the complete Bioanalytical method.

V Kiran Kumar et al [23], 2009, had reported a rapid and accurate reverse phase HPLC method developed for the estimation of Gefitinib in tablet dosage form. A Hypersil BDS RP C18, 250x4.6 mm, 5 µm particle size, with mobile phase consisting of 0.02 M Di-potassium Hydrogen ortho phosphate and Methanol in the ratio of 10:90 v/v was used. The flow rate was 1.0 ml/min and the effluents were monitored at 246 nm. The retention time was 3.7 min. The detector response was linear in the concentration of 25µ-300µg/ml. The respective linear regression

equation being Y= 94342.26x+77672.7. The limit of detection and limit of quantification was 0.125 µg/ml and 0.15 µg/ml respectively. The percentage assay of Gefitinib was 99.5 %. The method was validated by determining its accuracy, precision and system suitability. The results of the study showed that the proposed RP-HPLC method is simple, rapid, precise and accurate, which is useful for the routine determination of Gefitinib in bulk drug and in its pharmaceutical dosage form.

Ming Zhao et al [(24)], 2005; had developed and validated using liquid chromatography–tandem mass spectrometry (LC/MS/MS) for determination of gefitinib in human plasma and mouse plasma and tissue. Sample preparation involved a single protein precipitation step by the addition of 0.1 mL of plasma or a 200 mg/mL tissue homogenate diluted 1/10 in human plasma with 0.3 mL acetonitrile. Separation of the compounds of interest, including the internal standard (d8)-gefitinib, was achieved on a Waters X-Terra TM C_{18} (50 mm×2.1 mm i.d., 3.5 µm) analytical column using a mobile phase consisting of acetonitrile–water (70:30 v/v) containing 0.1% formic acid and isocratic flow at 0.15 mL/min for 3 min. The analytes were monitored by tandem mass spectrometry with electro spray positive ionization. Linear calibration curves were generated over the range of 1–1000 ng/mL for the human plasma samples and 5–1000 ng/mL for mouse plasma and tissue samples with values for the coefficient of determination of >0.99. The values for both within- and between-day precision and accuracy were well within the generally accepted criteria for analytical methods (<15%). This method was subsequently used to measure concentrations of gefitinib in mice following administration of a single dose of 150 mg/kg intraperitoneally and in cancer patients receiving an oral daily dose of 250 mg.

PVV Satyanarayana et al [(25)], 2011; had developed and validated for the estimation of Gefitinib in tablet dosage form. An Inertsil ODS C-18, 5 µm column having 250 x 4.6 mm internal diameter in isocratic mode with mobile phase containing acetonitrile: methanol: tetrahydrofuran in the ratio of 20:70:10 (v/v/v) was used. The flow rate was 1.0 ml/min and effluents were monitored at 251 nm. The retention time for Gefitinib was 4.282 min. The method was validated for linearity, accuracy, precision, specificity, limit of detection, limit of quantification and robustness. Limit of detection and limit of quantification were found to be

0.09 ppm and 0.29 ppm respectively and recovery of Gefitinib from tablet formulation was found to be 99.16%. The proposed method was successfully applied for the quantitative determination of Gefitinib in tablet formulation.

A Ratna Kumari et al [26], 2010; had reported for the quantitative estimation of Gefitinib in bulk drug and tablet dosage forms. Gefitinib is a drug that is used to treat several types of lung cancer and in particular, it is used alone for the treatment of patients with a specific type of lung cancer termed non - small cell lung cancer (NSCLC) that has not responded to chemotherapy. It works by preventing lung cancer cells from growing & multiplying. The drug exhibits absorption maximum at 252 nm in 0.1N HCL and obeys Beer's law in the concentration range of 10-35 µg/ml. The method was extended to pharmaceutical preparations and there is no interference from any common pharmaceutical additives.

Ling-zhi Wang et al [27], 2011; had developed LC-MS/MS method and validated for the simultaneous quantification of gefitinib and its predominant metabolite, O-desmethyl gefitinib in human plasma. Chromatographic separation of analytes was achieved on an Alltima C18 analytical HPLC column (150 mm × 2.1 mm, 5 µm) using an Isocratic elution mode with a mobile phase comprised acetonitrile and 0.1% formic acid in water (30:70, v/v). The flow rate was 300 µL/min. The chromatographic run time was 3 min. The column effluents were detected by API 4000triple quadrupole mass spectrometer using electro spray ionization (ESI) in positive mode. Linearity was demonstrated in the range of 5–1000 ng/mL for gefitinib and 5–500 ng/mL for O-desmethyl gefitinib. The intra- and inter-day precisions for gefitinib and O-desmethyl gefitinib were ≤10.8% and the accuracies ranged from 89.7 to 104.7% for gefitinib and 100.4 to 106.0% for O-desmethyl gefitinib. This method was used as a bioanalytical tool in a phase I clinical trial to investigate the possible effect of hydroxychloroquine on the pharmacokinetics of gefitinib. The results of this study enabled clinicians to ascertain the safety of the combination therapy of hydroxychloroquine and gefitinib in patients with advanced (Stage IIIB–IV) non-small cell lung cancer (NSCLC).

Lionel Faivre et al [28], 2011; had developed a simple and sensitive LC-MS/MS method to simultaneously quantify these two TKI in plasma. Following liquid–liquid extraction, gefitinib,

erlotinib and sorafenib (internal standard), were separated with gradient elution (on a C8+ Satisfaction using a mobile phase of acetonitrile/20 mM ammonium acetate pH 4.5). Samples were eluted at a flow rate of 0.4 ml/min throughout the 15-min run. Dual UV wavelength mode was used, with gefitinib and erlotinib monitored at 331 nm, and sorafenib at 249 nm. The calibration was linear in the range 20–1000 ng/ml and 80–4000 ng/ml for gefitinib and erlotinib, respectively. Inter- and intra-day imprecision were less than 7.2% and 7.6% for gefitinib and erlotinib, respectively. This analytical method was successfully applied to assess the steady state plasma exposure to these TKI in NSCLC patients. This simple, sensitive, accurate and cost-effective method can be used in routine clinical practice to monitor gefitinib or erlotinib concentrations in plasma from NSCLC patients.

Madireddy Venkataramanna et al [(29)], had established a degradation pathway for gefitinib as per ICH recommendations by validated and stability indicating reverse phase liquid chromatographic method. Gefitinib is subjected to stress conditions of acid, base, oxidation, thermal and photolysis. Significant degradation is observed in acid and base stress conditions. Two impurities are studied among which one impurity is found prominent degradant. The stress samples are assayed against a qualified reference standard and the mass balance is found close to 99.5%. Efficient chromatographic separation is achieved on a Agilent make XDB-C18, 50 × 4.6 mm with 1.8 µm particles stationary phase with simple mobile phase combination delivered in gradient mode and quantification is carried at 250 nm at a flow rate of 0.5 mL·min–1. In the developed RPLC method the resolution between gefitinib and the potential impurities is found to be greater than 5.0. Regression analysis shows an r value (correlation coefficient) of greater than 0.998 for gefitinib and the two potential impurities. This method is capable to detect the impurities of gefitinib at a level of 0.01% with respect to test concentration of 0.5 mg/mL for a 4 µL injection volume. The developed RRLC method is validated with respect to specificity, linearity & range, accuracy, precision and robustness for impurities determination and assay determination.

Sandra Roche et al [(30)]**, 2009;** had developed a liquid chromatography tandem mass spectrometry (LC–MS/MS) method to quantify cellular levels of the tyrosine kinase inhibitors

(TKIs) dasatinib (SprycelTM) and lapatinib (TykerbTM, TyverbTM). Cellular samples were extracted with tetra-butyl methyl ether: acetonitrile (3:1, v/v):1M ammonium formate pH 3.5 (8:1, v/v) mixture. Separation was achieved on a Hyperclone BDS C_{18} (150mm×2.0mm 3 µm) column with isocratic elution using a mobile phase of acetonitirile–10 mM ammonium formate, pH 4 (54:46, v/v), at a flow rate of 0.2 mL/min. The TKIs were quantified using a triple quadrupole mass spectrometer which was operated in multi reaction-monitoring mode employing positive electro spray ionization. The limit of detection and limit of quantification for lapatinib was determined to be 15 and 31 pg on column, respectively. The limit of detection and quantification for dasatinib was 3 and 15 pg on column, respectively. The method allowed for sensitive and accurate determination of cellular levels of dasatinib and lapatinib. In addition, we examined the potential for this method to be utilized to quantitate other TKIs, using gefitinib, erlotinib, imatinib and sorafenib as examples. In principle, these agents were also quantifiable by this method, however, no drug specific validation studies were undertaken with these TKIs. The data indicates that in the cancer cell-line model, DLKP, significantly more lapatinib accumulates in cells in comparison to dasatinib. Additionally, over-expression of the membrane protein drug transporter, P-glycoprotein (P-gp) a common cancer drug resistance mechanism, greatly reduces the cellular accumulation of dasatinib but not of lapatinib.

N. Y. Sreedhar et al [(31)], 2011; had developed an ADSV method for Gefitinib which is a drug that is used to treat several types of lung cancer and in particular, it is used alone for the treatment of patients with a specific type of lung cancer termed non-small cell lung cancer (NSCLC). An adsorptive stripping voltammetric method was developed for the quantitative analysis of gefitinib gives a peak at -0.78 V at HDME. From the structural point of view gefitinib contains a > C=N- moiety which can be electrochemically reduced at universal buffer (pH 4.0). Millicoulometric experiment is performed successfully in estimating the number of electrons and proton to understand reduction mechanism. The adsorptive stripping voltammetric peak was adequately well resolved, reproducible and linear dependent with the gefitinib concentration. For quantification the calibration plot method for gefitinib concentrations ranging between 1.0×10^{-4} M to 1.0×10^{-8} M at pH 4.0 was elected. The proposed ADSV method was successfully applied to the determination of Gefitinib in pharmaceutical formulations and urine samples.

Yousef F. M. Alqahtani et al [32], 2009; had developed a method which is based on First Order derivative spectrophotometry of the colored product which was formed by charge transfer complexation of Sertraline (n-donor) with π-acceptor chloranilic acid. First derivative spectrophotometry has been evaluated by measuring of the derivative signal at 475.72 nm – 588.40 nm (peak to peak amplitude). Calibration graph was established for 5-100 µg.mL-1 of Sertraline with main percentage recoveries. The proposed method was applied successfully to the determination of Sertraline in pharmaceutical tablet with good accuracy and precision.

Krishna R Gupta et al [33], 2009; had developed three UV methods for the estimation of Diacerein in Pharmaceutical Formulations. Diacerein has the maximum absorbance at 256.2 nm in zero order spectra formed the basis for method A while first order derivative spectrum showed peak at 250.0 nm when n = 1 for method b and method C applied was Area under Curve (AUC) in wavelength range of 262.0-250.0 nm. Method A utilizes A1%, 1cm value at λ_{max} for its analysis. Calibration curve (Regression equation) was used for method B and C for analysis of Diacerein respectively. Drug was found to obey the Beer-Lambert's law in the concentration range of 5-30 µg/mL in all three proposed methods. Results of the analysis were validated statistically and by recovery studies. Results were found to be satisfactory and can be adopted for routine analysis of the drug.

Angel M. Carcaboso et al [34], 2010; had performed intracerebral microdialysis on mice bearing orthotopic human gliomas (U87 and MT330) and assessed topotecan tumor ECF (tECF) penetration and the effect of gefitinib on topotecan tECF penetration and intratumor topotecan distribution. We found that topotecan penetration (Ptumor) of U87 was 0.96 ± 0.25 (n = 7) compared with that of contralateral brain (Pcontralateral, 0.42 ± 0.11, n = 5; P = 0.001). In MT330 tumors, Ptumor (0.78 ± 0.26, n = 6) and Pcontralateral (0.42 ± 0.11, n = 5) also differed significantly (P = 0.013). Because both tumor models had disrupted blood-brain barriers and similar Ptumor values, we used U87 and a steady-state drug administration approach to characterize the effect of gefitinib on topotecan Ptumor. At equivalent plasma topotecan exposures, we found that Ptumor after gefitinib administration was lower. In a separate cohort of animals, we determined the volume of distribution of unbound topotecan in tumor (Vu,tumor)

and found that it was significantly higher in groups receiving gefitinib, implying that gefitinib administration leads to a greater proportion of intracellular topotecan. Our results provide crucial insights into the role that transporters play in central nervous system drug penetration and provide a better understanding of the effect of co-administration of transporter modulators on anticancer drug distribution within a tumor.

CHAPTER -3

Drug profile

DRUG PROFILE [35]

CHEMICAL PROFILE:-

Molecular formula : $C_{22}H_{24}ClFN_4O_3$

Molecular weight : 446.9

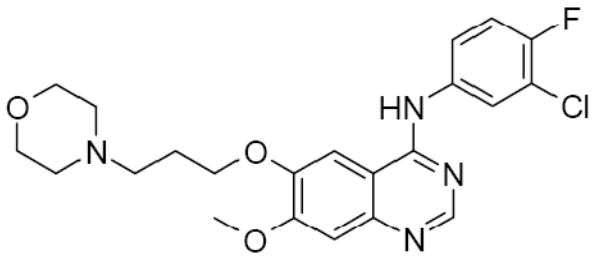
Description : White Colored powder

Solubility : Soluble in both methanol and ethanol and

Sparingly soluble in tetra hydro furan

Storage Conditions : Store at controlled room temperature 20-25ºc (68-77°F)

Structure :



Category : Novel Anti-emetic agent used in Cancer Chemotherapy.

PHARMACOKINETIC DATA:

Bioavailability : 60%

Metabolism :

Gefitinib undergoes extensive hepatic metabolism in humans, predominantly by CYP3A4. Three sites of biotransformation have been identified. Metabolism of the N-propoxymorpholino group, demethylation of the methoxy substituent on the quinazoline, and oxidative defluorination of the halogenated phenyl group.

Excretion :

Excretion was primarily via the faeces (>90% of recovered dose) oral and intravenous dosing in rats, rabbits and dogs. Elimination was relatively slow. The proportion of the dose being recovered in the first 24 hours was 70% in rats, 51%in rabbits and 40% in dogs.

Chemical IUPAC Name:

Gefitinib is an anilinoquinazoline with the chemical name of 4-Quinazolinamine, N-(3-chloro-4-fluorophenyl)-7-methoxy-6-[3-4-morpholin propoxy].

PHARMACOLOGICAL PROFILE [36]:

Mechanism of Action:

The mechanism of the clinical antitumor action of gefitinib is not fully characterized. Gefitinib inhibits the intracellular phosphorylation of numerous tyrosine kinases associated with trans membrane cell surface receptors, including the tyrosine kinases associated with the epidermal growth factor receptor (EGFR-TK). EGFR is expressed on the cell surface of many normal cells and cancer cells.

No clinical studies have been performed that demonstrate a correlation between EGFR receptor expression and response to gefitinib.

Adverse Reactions:

The most common adverse drug reactions reported at the recommended 250 mg daily dose, occurring in more than 20% of patients, are diarrhoea, sometimes associated with dehydration and mainly mild or moderate in nature and less commonly, severe ; and skin reactions (including rash, acne, dry skin and pruritus). Approximately 10% of patients had a severe ADR. Approximately 3% of patients stopped therapy due to an ADR. The onsets of these ADRs usually occurred within the first month of therapy and were generally mild and non-cumulative as well as reversible.

Indications and Usage:

Gefitinib is indicated as monotherapy for the continued treatment of patients with locally advanced or metastatic non-small cell lung cancer after failure of both platinum-based and docetaxel chemotherapies who are benefiting or have benefited from Gefitinib.

In light of positive survival data with other agents including another oral EGFR inhibitor, physicians should use other treatment options in advanced non-small cell lung cancer patient populations who have received one or two prior chemotherapy regimens and are refractory or intolerant to their most recent regimen.

The effectiveness of Gefitinib was initially based on objective response rates. Subsequent studies intended to demonstrate an increase in survival have been unsuccessful. Specifically, results from a large placebo-controlled randomized trial in patients with advanced NSCLC who progressed while receiving or within 90 days of the last dose of chemotherapy or were intolerant to the most recent prior chemotherapy regimen, did not show an improvement in survival.

Drug Interactions

Substances that are inducers of CYP3A4 activity increase the metabolism of gefitinib and decrease its plasma concentrations. In patients receiving a potent CYP3A4 inducer such as rifampicin or phenytoin, a dose increase to 500 mg daily should be

considered in the absence of severe adverse drug reaction, and clinical response and adverse events should be carefully monitored.

How supplied

IRESSA tablets are supplied as round, biconvex, brown film-coated tablets intagliated with "IRESSA 250" on one side and plain on the other side, each containing 250 mg of gefitinib.

Dosage and Administration:

The recommended daily dose of IRESSA is one 250 mg tablet with or without food. Higher doses do not give a better response and cause increased toxicity.

For Patients who have Difficulty Swallowing Solids -

IRESSA tablets can also be dispersed in half a glass of drinking water (non-carbonated). No other liquids should be used. Drop the tablet in the water, without crushing it, stir until the tablet is dispersed (approximately 10 minutes) and drink the liquid immediately. Rinse the glass with half a glass of water and drink. The liquid can also be administered through a naso-gastric tube.

CHAPTER -4

AIM & OBJECTIVE

AIM AND OBJECTIVE

Estimation of drugs in biological media is increasingly important nowadays, which reveals information like bioavailability, bioequivalence, drug abuse, and pharmacokinetics and drug research.

HPLC is the most suitable technique for the analysis of biological fluids owing to its well-developed characteristics and ruggedness. It is an extremely sensitive, precise, accurate, and rapid, separation technique.

For the estimation of Gefitinib present in tablet dosage form HPLC and First Derivative Spectroscopic methods are considered to be the most suitable. These methods are powerful, extremely precise, accurate, sensitive, specific, linear and rapid in analyzing the sample. The chromatographic method was found to be most suitable than the UV-Spectroscopic due to its very high sensitivity.

Gefitinib is an anti-neoplastic agent mostly used for cancer patients in long-term chemotherapy. As Gefitinib has nephrotoxicity it is necessary to assess the blood level concentration and toxicological interpretation through therapeutic drug monitoring. So, there is a need to develop simple, rapid, reliable and cost effective analytical method for the pharmacokinetic study and clinical routine monitoring.

From the extensive literature review it is found that less works have been reported on estimation of Gefitinib in serum by LC-MS/MS and also a very few studies have been reported on bio analytical method development in human plasma.

The present study is planned to develop newer analytical method for the determination of Gefitinib in tablet dosage form by First Derivative Spectroscopy and Bio-Analytical method development in human plasma by RP-HPLC under different chromatographic conditions.

The reasons for developing First Derivative Spectroscopic method for determination of Gefitinib in tablet dosage form and Bio-Analytical method by RP-HPLC are as follows:

- No First Derivative Spectroscopic method was available for the estimation of this drug.
- To develop newer RP-HPLC Method by Isocratic mode.
- To reduce the run time.
- To carry out estimation of Gefitinib by using different chromatographic conditions.

CHAPTER -5

PLAN OF WORK

PLAN OF WORK

1. HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD:

1.1 Bioanalytical method development for Gefitinib:

a. Optimization of chromatographic conditions

- Selection of wavelength
- Selection of initial separation conditions
- Selection of mobile phase (pH, peak modifier, solvent strength, ratio and flow rate)
- Nature of the stationary phase
- Selection of internal standard
- Estimation of Gefitinib

b. Validation of the method

The developed method were proposed to be validated using the various validation parameters such as,

- Accuracy
- Precision
- Linearity
- Lower Limit of quantitation (LLOQ)
- Selectivity / Specificity
- System suitability
- Ruggedness

2. FIRST DERIVATIVE SPECTROSCOPIC METHOD :

Gefitinib possess light absorption character in UV region. Hence, this method is based on the measurement of light absorption in UV region using mobile phase (methanol) as a solvent.

2.1 Validated Derivative Spectrophotometric estimation of Gefitinib in a tablet dosage form was performed as follows:

- Selection of suitable solvent
- Determination of λ_{max} of drug
- Determination of linearity range of drug
- Selection of derivative spectroscopic method
- Selection of sampling wavelengths
- Estimation of formulation
- Method validation

CHAPTER -6

METHODOLOGY

METHODOLOGY

1. UV SPECTROSCOPY

1.1 First order derivative spectroscopy

1.1.1 Selection of analytical wavelength:

Dilutions were prepared for drug from the standard stock solution and the solutions were scanned in the wavelength range of 200-400 nm. The absorption spectra **Fig : 1** thus obtained was derivatized from first order. The first order derivative spectrum was selected for the analysis of the drugs.

1.1.2 Selection of solvent

The solvent used in spectrophotometry must meet the certain requirements to assure successful and accurate results. The chosen solvent must dissolve the sample and yet it has to be compatible with the cuvette materials.

The solvent must also be relatively transparent in the spectral region of interest and should be of consistent purity (spectrophotometry grade solvents used).

The chosen solvent must have good solublising power. Stable interaction with absorbing species.

For the estimation of gefitinib by first order spectrophotometry methanol is used as a suitable solvent

1.1.3 Preparation of stock solutions:

Standard Gefitinib 10 mg was weighed and transferred to a 10 ml volumetric flask and dissolved in methanol. The flask was shaken and volume was made up to the mark with methanol to give a solution containing 1000 µg / ml. From this stock solution, pipetted out

1 ml and placed into 10 ml volumetric flask. The volume was made up to mark with methanol to give a solution containing 100 µg / ml.

1.1.4 Selection of analytical concentration ranges:

From the standard stock solution of Gefitinib, appropriate aliquots were pipetted out in to 10 ml volumetric flasks and dilutions were made with methanol to obtain working standard solutions of concentrations from 2 to 50 µg / ml. Absorbance for these solutions were measured at 242 nm. For the standard solution analytical concentration range were found to be 2-12 µg / ml and those values were reported in **Table: 7.1.**

1.1.5 Calibration curve for the Gefitinib (2 – 12 µg / ml):

Appropriate volume of aliquots from standard Gefitinib stock solutions were transferred to different volumetric flasks of 10 ml capacity. The volume was adjusted to the mark with methanol to obtain concentrations of 2, 4, 6, 8, 10 and 12 µg / ml. Absorbance spectra of each solution against methanol as blank were measured at 242 nm and the graphs of absorbance against concentration was plotted and are shown in **Fig: 15.** The regression equation and correlation coefficient was determined and are presented in **Table: 7.2.**

1.1.6 Sample preparation for determination of Gefitinib from dosage form:

Ten tablets (Gefiticip) were weighed and finely powdered. The powder equivalent to 10 mg of Gefitinib was accurately weighed and transferred to volumetric flask of 10 ml capacity containing 2.5 ml of the methanol and sonicated for 10 min. The flask was shaken and volume was made up to the mark with methanol to give a solution of 1000 µg / ml. Carefully filtered through Whatmann filter paper (No. 41) and used for the estimation of Gefitinib.

1.2 Validation of Spectrophotometric method

1.2.1 Linearity and Range:

The linearity of analytical method is its ability to elicit test results that are directly proportional to the concentration of analyte in sample with in a given range. The range of analytical method is the interval between the upper and lower levels of analyte that have been demonstrated to be determined within a suitable level of precision, accuracy and linearity.

1.2.2 Precision:

The precision of an analytical method is the degree of agreement among individual test results, when the method is applied repeatedly to multiple samplings of homogenous samples. It provides an indication of random error results and was expressed as coefficient of variation (CV).

1.2.3 Intra and inter-day precision:

Variations of results within the same day (intra-day), variation of results between days (inter-day) were analyzed. Intra-day precision was determined by analyzing Gefitinib for six times in the same day at 242 nm. Inter-day precision was determined by analyzing daily once for six days at 242 nm and % CV was calculated and were shown in **Table: 7.3.**

1.2.4 Accuracy:

Accuracy is the closeness of the test results obtained by the method to the true value. To study the accuracy, 20 tablets were weighed and powdered and analysis of the same was carried out. Recovery studies were carried out by adding known amount of standard drug solution (9 or 10 µg / ml) to the sample solution. The % recovery was calculated and reported in **Table: 7.4.**

1.2.5 Ruggedness:

The solutions were prepared and analyzed with change in the analytical conditions like different laboratory conditions and different analyst and reported in **Table: 7.5.**

2. HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

2.1 Materials and Instruments used

2.1.1. Drug sample and Study products-

Gefitinib was procured from NATCO PHARMA LIMITED, Hyderabad, Andhra Pradesh.

Plasma: Blank plasma was collected from Kovai Medical Center and Hospitals, Coimbatore.

2.1.2. Chemicals and solvents used for estimation-

- HPLC Water - Qualigens, Mumbai, India.
- Acetonitrile - Rankem, Mumbai, India.
- Methanol - Himedia, Mumbai, India.
- Distilled water - Double Distilled water.
- Potassium Di-hydrogen orthophosphate - Analytical grade, SD fine chemicals, Mumbai.
- Tri-Ethyl Amine (TEA) - Analytical grade, SD fine chemicals, Mumbai.
- Orthophosphoric acid - SD fine chem. Ltd, Mumbai.

2.1.3**. Instruments used:**

- ✓ Elico pH meter LI 127.
- ✓ Shimadzu LC-20 AT HPLC.
- ✓ SPD-M20A Prominence diode array detector.
- ✓ Shimadzu 1700 LC-UV Spectrophotometer.
- ✓ Sonica ultrasonic cleaner.
- ✓ Solvent filtration unit – Millipore.
- ✓ Shimadzu electronic balance AY 220.
- ✓ Ultra cooling centrifuge – Remi, India

2.2. OPTIMIZATION OF CHROMATOGRAPHIC CONDITION FOR THE ESTIMATION OF GEFITINIB[37]

2.2.1. Selection of Wavelength-

An UV spectrum of 10 µg/ml Gefitinib in methanol was recorded by scanning in the range of 200 nm to 400 nm. A wavelength which gives good response for the drugs to be detected is to be selected. From the UV spectrum a wavelength of 332 nm was selected. Gefitinib showed optimal absorbance at this wavelength.

2.2.2. Selection of chromatographic method

Selection of proper chromatographic method depends on the nature of the sample or its properties like ionic/ionizable/neutral character, its molecular weight and solubility. The drug selected for the present study was polar in nature hence, reverse phase HPLC or ion-pair or ion–exchange chromatography method must be used. Because of its simplicity and suitability for initial separations reverse phase method was selected.

2.2.3. Initial chromatographic conditions for separation of Gefitinib Standard solution:

10µg/ml of Gefitinib was prepared by dissolving in 10 ml of HPLC grade methanol.

Equipment

System : Shimadzu gradient HPLC

Pump : LC-20AT prominence solvent delivery system

Detector : SPD-M20A prominence Diode array detector

Injector : Rheodyne 7725i with 20µl loop

Chromatographic Conditions – 1

Stationary phase	: Phenomenex C18 column
Mobile phase	: Solvent A – Triethylamine (TEA) 0.5% pH adjusted to 3.5 (with Orthophosphoric acid) Solvent B - Acetonitrile
Solvent ratio	: 50: 50 (A: B)
Detection Wavelength	: 332 nm
Flow rate	: 1.0 ml/min
Sample size	: 20 µl
Needle wash	: HPLC grade water
Temperature	: Room temperature (25ºC)

At the above chromatographic conditions Gefitinib was eluted at the retention time of 2.2 min. The peak observed was broad and asymmetric, thus not selected further for studies.

Chromatographic Conditions – 2

Stationary phase	: Phenomenex C18 column
Mobile phase	: Solvent A – Triethylamine (TEA) 0.5%
	pH adjusted to 4 (with Orthophosphoric acid)
	Solvent B - Acetonitrile
Solvent ratio	: 80: 20 (A: B)
Detection Wavelength	: 332 nm
Flow rate	: 1.0 ml/min
Sample size	: 20 µl
Needle wash	: HPLC grade water
Temperature	: Room temperature (25ºC)

Gefitinib was eluted at retention time of 2.9 minute with peak splitting and fronting, hence not selected further for method development.

Chromatographic Conditions – 3

Stationary phase	: Phenomenex C18 column
Mobile phase	: Solvent A – Phosphate buffer 20mM
	pH adjusted to 3.5 (with Orthophosphoric acid)
	Solvent B - Acetonitrile
Solvent ratio	: 50: 50 (A: B)
Detection Wavelength	: 332 nm
Flow rate	: 1.0 ml/min
Sample size	: 20 µl
Needle wash	: HPLC grade water
Temperature	: Room temperature (25ºC)

Gefitinib was eluted at retention time 3.5 minutes with peak tailing and no peak was observed for internal standard.

Chromatographic Conditions – 4

Stationary phase : Phenomenex C18 column

Mobile phase : Solvent A – Phosphate buffer 10 mM
pH adjusted to 4.5 (with Orthophosphoric acid)
Solvent B - Acetonitrile

Solvent ratio : 70: 30 (A: B)

Detection Wavelength : 332 nm

Flow rate : 1.0 ml/min

Sample size : 20 µl

Needle wash : HPLC grade water

Temperature : Room temperature (25ºC)

Gefitinib was eluted at retention time of 2.8 minutes with fronting and no peak was observed for internal standard, hence, not selected further for method development.

Chromatographic Conditions – 5

Stationary phase : Phenomenex C18 column

Mobile phase : Solvent A – Heptane sulphonic acid 0.05%
pH adjusted to 4.5 (with Orthophosphoric acid)
Solvent B - Acetonitrile

Solvent ratio : 50: 50 (A: B)

Detection Wavelength : 332 nm

Flow rate : 1.0 ml/min

Sample size : 20 µl

Needle wash : HPLC grade water

Temperature : Room temperature (25ºC)

Gefitinib was eluted at retention time of 4.1 minutes with peak tailing, hence not selected further for method development.

Chromatographic Conditions – 6

Stationary phase : Phenomenex C18 column

Mobile phase : Solvent A – Phosphate buffer of 20 mM (99.5ml) and TEA (0.5ml) pH adjusted to 6.5 (with Orthophosphoric acid)

Solvent B - Acetonitrile

Solvent ratio : 50: 50 (A: B)

Detection Wavelength : 332 nm

Flow rate : 1.0 ml/min

Sample size : 20 µl

Needle wash : HPLC grade water

Temperature : Room temperature (25ºC)

Gefitinib was eluted at 8.8 mins and internal standard at 6.1mins with perfect peak properties, hence selected for further studies.

2.2.4. Effect of pH

Using 50% of acetonitrile and buffer solution of different pH ranging from 3.5, 4.5, 6.5 at 332 nm, the standard solution was runned up to 20 min at a flow rate of 1.0 ml/min, 1.0 ml/min and 1.1 ml/min. The retention time of Gefitinib was 3.5, 4.1, 8.8minutes respectively. For the present study, a pH of 6.5 was selected as the chromatogram obtained with this pH was symmetrical in shape.

2.2.5. Effect of ratio of mobile phase

Mixture of Phosphate buffer and TEA and acetonitrile with 50:50, 70:30 and 80:20 ratios were used as the mobile phase. At 50:50 ratios, symmetric peaks were eluted at 6.1 mins and 8.8 mins for internal standard (Duolextine Hcl) and Gefitinib respectively. At 70:30 and 80:20 ratios the peaks were asymmetrical in shape. Thus for the present study 50:50 ratio of Phosphate buffer + TEA and acetonitrile was selected as the mobile phase.

2.2.6. Effect of Peak Modifier

With the above chromatographic conditions, to improve the peak shapes and separation 0.5ml of Triethylamine (TEA) was added and chromatogram was recorded. The retention time of gefitinib was found to be 8.8mins.

2.2.7. Effect of flow rate

Keeping the mobile phase ratio at (50:50, v/v) Phosphate buffer + TEA: acetonitrile, the chromatograms were recorded at a flow rate of 0.5ml/min, 1.0ml/min 1.1ml/min. At flow rate of 1.0ml/min, the peaks were sharp and separated with good resolution. Hence, 1.0ml/min was kept constant for the present analysis.

2.2.8. Selection of internal standard

Based upon polarity and solubility, Tinidazole, Ambroxol, Lamivudine and Duolextine Hcl were selected and chromatographed along with the standard drug. The elution time of Duolextine Hcl was 6.1 min. The peak of Duolextine Hcl was symmetric and well resolved from the peak of the Gefitinib. Hence, for the present study Duolextine Hcl was selected as the internal standard.

2.2.9. Fixed chromatographic conditions

The following chromatographic conditions were used for the estimation of Gefitinib in human plasma.

Stationary phase	: Phenomenex C18 column
Mobile phase	: Solvent A – Phosphate buffer of 20mM (99.5ml) and TEA (0.5ml) pH adjusted to 6.5 (with Orthophosphoric acid) Solvent B - Acetonitrile
Solvent ratio	: 50: 50 (A: B)
Detection Wavelength	: 332 nm
Flow rate	: 1.0 ml/min
Sample size	: 20 µl
Needle wash	: HPLC grade water
Temperature	: Room temperature (25ºC)

2.2.10. Pretreatment method for biological fluid:

Method of sample preparation is an important criterion for biological samples. For the present study plasma was obtained from Clinical Laboratory Services of KMCH Hospital, Coimbatore. Protein precipitation method was selected for the present study.

Protein precipitation

This method is the most commonly used method for the extraction of analytes from biological fluids. In this method precipitating agents like acetonitrile, methanol mixture of acetonitrile-methanol etc was added to the blank plasma to precipitate the proteins present in the plasma and then the precipitate formed was removed by filtration or centrifugation. The supernatant resulted is directly injected into HPLC column without any further treatment and chromatograms were recorded.

An aliquot of 0.1ml of plasma in a glass tube 0.05 ml of standard solution, 0.05 ml of internal standard solution and 0.2 ml of mixture of acetonitrile-methanol in the ratio of (50:50 v/v) was added and then centrifuged for 15 minutes at 5000 rpm. The same extraction procedure was also repeated by using methanol and acetonitrile separately for extraction of drug from plasma and the percentage recovery was calculated for all the precipitating agents.

The chromatograms of plasma extracted with acetonitrile, methanol and mixture of acetonitrile- methanol (50:50, v/v) were recorded using the fixed chromatographic conditions. The chromatogram of blank plasma without any drug was also recorded. Based up on the percentage recovery acetonitrile-methanol mixture (50:50, v/v) was selected for the present study because of its higher percentage recovery.

2.2.11. Preparation of standard stock solution:

Stock solution of Gefitinib and internal standard 1mg/ml were prepared separately by dissolving 10mg of each drug in 10ml standard flasks and the volume was made up to 10 ml with the mobile phase.

2.2.12. Working standards:

From the stock solution working standard solutions of 100µg/ml was prepared by diluting 1ml to 10ml with mobile phase. Further solutions were made by from the above solution by diluting 0.4ml, 0.8ml, 1.2ml, 1.6ml, and 2.0ml standard solutions to 10ml in a standard flask with mobile phase. An aliquot of 0.05ml from these working standards were taken in serial 10ml standard flasks to effect concentrations of 100, 200, 300, 400, and 500ng/ml respectively. In the similar way the working standards were prepared for internal standard also.

2.2.13. Preparation of standard graph:

Preparation of calibration standards

To 0.1ml of blank plasma 0.05ml of working standard solution of Gefitinib and 0.05ml of internal standard working solutions were added to get concentration of 100, 200, 300, 400, and 500ng/ml respectively. To these calibration standards 0.2ml of precipitating agent mixture of acetonitrile-methanol (50:50, v/v) was added and then centrifuged for 15 minutes at 5000 rpm. After centrifugation the clear supernatant liquid was collected and a quantity of 20µl was injected into the HPLC column and chromatograms were recorded. Standard calibration graph was plotted using ratio of peak area of Gefitinib to its concentration.

2.3. ESTIMATION OF GEFITINIB IN HUMAN PLASMA:

Recording the chromatogram

The optimized chromatographic conditions were maintained to record the chromatograms of the calibration standards of Gefitinib and sample from a clinical study. First, baseline stabilization was done for about 20 minutes. Then standard solutions, calibration standard solutions and sample from clinical study containing Gefitinib were injected and chromatograms were recorded.

2.4. VALIDATION OF THE METHOD

After developing a method its validation is necessary to prove the suitability of the method for the intended purpose. Here the procedure followed for the validation of the developed method is described.

a) Precision*:*

Intraday and interday precision studies were conducted. In intraday precision plasma sample containing drug at three different concentrations with internal standard were injected and chromatogram was recorded. Similarly interday precision over a two week period time was evaluated.

Acceptance criteria:

RSD of the mean concentration of five readings should be less than 15% for bioanalytical method.

b) Accuracy:

It is the closeness of mean tests results obtained by the method to true concentration of analyte. It is also named as trueness. In this studies the selected concentration of the plasma were injected six times and mean peak area for each concentration was calculated. Concentration of the each injection was calculated and the standard deviation between the readings is calculated. To bioanalytical study the percentage RSD should be less than 15%.

c) Recovery studies:

The relative recovery of drug from plasma was calculated by comparing the readings of concentration obtained from the drug spiked plasma to that of equal concentration from standard sample. Recovery studies were carried out six times for sample concentration at three levels within the calibration curve.

Acceptance Criteria:

For an assay method, mean recovery should be 85-105% ± 2%.

d) Linearity and Range:

Linearity and range were estimated by using calibration curve. By using calibration standards prepared by spiking plasma (Gefitinib) and internal standard (Duolextine Hcl) at different concentrations like 100ng/ml to 500ng/ml the calibration graph was plotted taking concentration of spiked plasma on x-axis and peak area on y-axis. The linearity is determined from 50% to 150% of the proposed concentration.

Acceptance Criteria:

Coefficient of correlation of the calibration should be not less than 0.99

e) Lower Limit of Quantification (LLOQ):

The LLOQ is determined by using the calibration curve. Limit of quantitation is the concentration of substance in the sample that will give a signal-to-noise ratio of 10:1. Detection limit corresponds to the concentration that will give a signal-to-noise ratio of 3:1. The signal to noise ratio were performed by comparing measured signal of blank plasma sample with those of known low concentration of drug.

f) Specificity:

Specificity of the method was demonstrated by using diode array detector peak purity test. The diode array spectrum of both standard and sample peak were recorded and compared. The other way for doing specificity based in measurement of absorbance ratio of drug peaks at two different levels. The retention time (Rt), resolution factor (Rs) and tailing (T) were noted for the peaks of Gefitinib. Peak purity study is done to prove that a developed method is specific for the drug of interest.

Acceptance criteria:

Purity angle should be less than purity threshold i.e.0.99-1.00

g) Stability:

Stability of the sample, standard and reagent used in HPLC method is required for a reasonable time to generate reproducible and reliable results. Stability of plasma sample spiked with drug were subjected to three freeze-thaw cycles, short term stability at room temperature for 3 hours and long term stability at -20^0C for four weeks. In addition, stability of standard solution and internal standard were performed at room temperature for 6 hours and under frozen condition for two weeks. The stability of this solution was studied by performing the experiment and looking for changes in separation, retention and asymmetry of the peak which were then compared with the pattern of chromatogram of freshly prepared solutions.

h) Selectivity:

Selectivity is the analytical method ability to differentiate and quantify the analyte in the presence of other components in the sample. The selectivity was established by two different methods.

Method I: Chromatograms of six blank plasma samples were compared with chromatogram obtained from standard solutions. Each chromatogram was tested for interferences due to endogenous plasma component on the retention times of the selected drugs.

Method II: This method involves the peak purity test method using diode array detector. The PDA spectrum, UV spectrum, absorbance ratio curve and first derivative spectrum of the standard and sample peaks was recorded using PDA detector and compared for the peak purity of drug.

i) Robustness:

The robustness of the method was studied by changing the chromatographic conditions slightly. The standard solutions were injected in these changed chromatographic conditions.

- ± 1 % difference in the ratio of acetonitrile in the mobile phase.
- ± 0.5 difference in units of pH of the buffer.

- ± 1 % difference in flow rate of the mobile phase.

In these changed conditions the separation factor, retention time and peak symmetry was calculated. Deviation in results from original run should be less than 2%.

j) System suitability studies:

In system suitability studies certain parameters were calculated namely, column efficiency, resolution, capacity factor by repeated injection of standard solutions. As specified in the USP these systems suitability studies were carried out.

Capacity factor (k') it is measurement of sample molecule how good is retained by a column during separation. The ideal k value ranges from 2-10.

$$\text{Capacity Factor (k')} = V_1 - V_0 / V_0$$

Where, V_1 is the retention volume at the apex of the peak (solute) and V_0 is the void volume of the system.

Resolution (Rs) is the difference between the retention times of two solutes divided by their average peak width. The ideal value of (Rs) is 1.5

$$\text{Resolution (Rs)} = Rt_1 - Rt_2 / 0.5(W_1 - W_2)$$

Where, Rt_1 and Rt_2 are the retention times of component 1 and 2, respectively.

Column Efficiency (N) of a column is measured by the number of theoretical plates per meter. For ideal good separation, column efficiency N value ranging from 5,000 to 100,000 plates/meters.

$$\text{Column efficiency (N)} = R_t^2 / W^2$$

Where R_t is the retention time and W is the peak width.

Peak asymmetry factor- For better column performance it was calculated by the formula. When asymmetry factor of value 0.9 to 1.1 then it is achievable for a well packed column.

$$\text{Peak asymmetry factor (As)} = b / a$$

Where a and b are the distances on either side of the peak midpoint.

System Suitability Parameters and Recommendations:

Parameter	Recommendation
Capacity Factor (k')	The peak should be well-resolved from other peaks and the void volume, generally k' > 2.0
Repeatability	RSD < / = 1 % for N > / = 5 is desirable.
Relative retention	Not essential as long as the resolution is stated.
Resolution (R_s)	R_s of > 2 between the peak of interest and the closest eluting potential interferent (impurity, excipient, degradation product, internal standard, etc.)
Tailing Factor (T)	T of < / = 2
Theoretical Plates (N)	In general should be > 2000

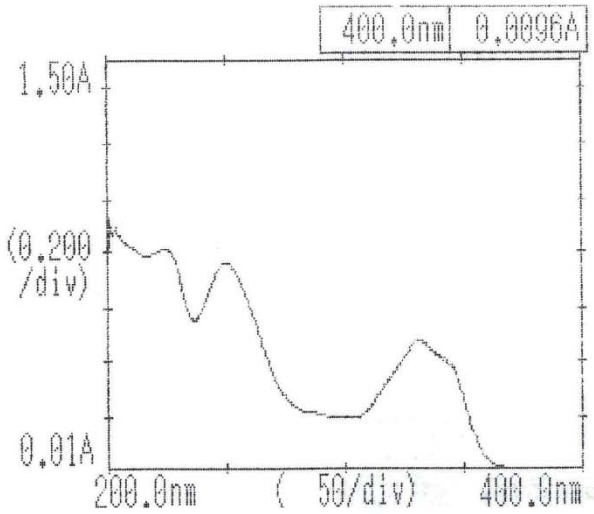


Fig : 1 – UV Spectra of Standard Gefitinb

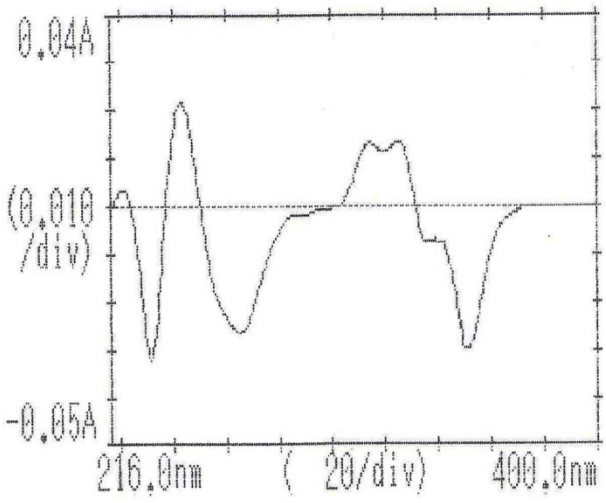


Fig : 2 – First order spectra of Gefitinib at ***242nm***

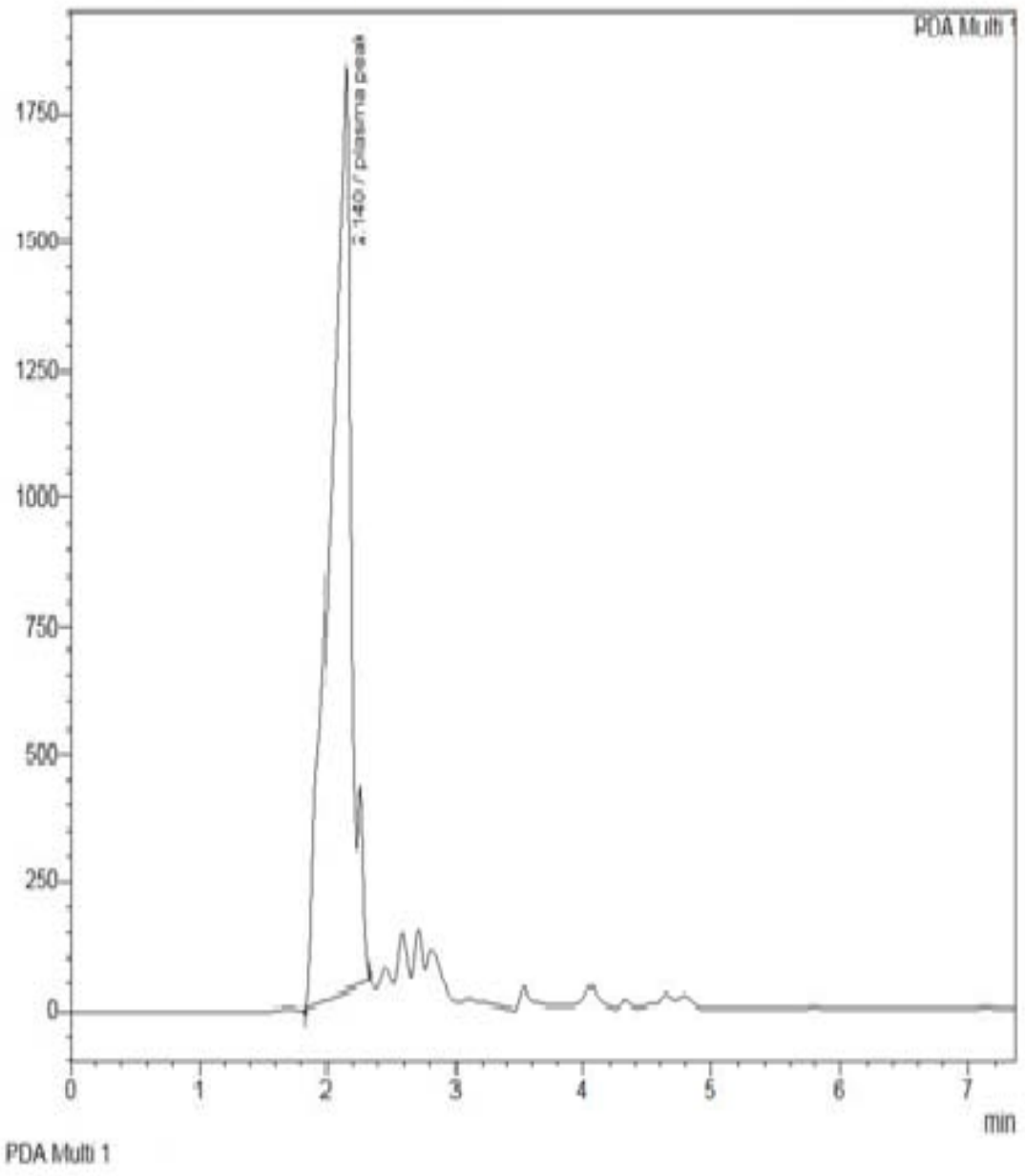


Fig : 3 - Chromatogram of Blank Plasma

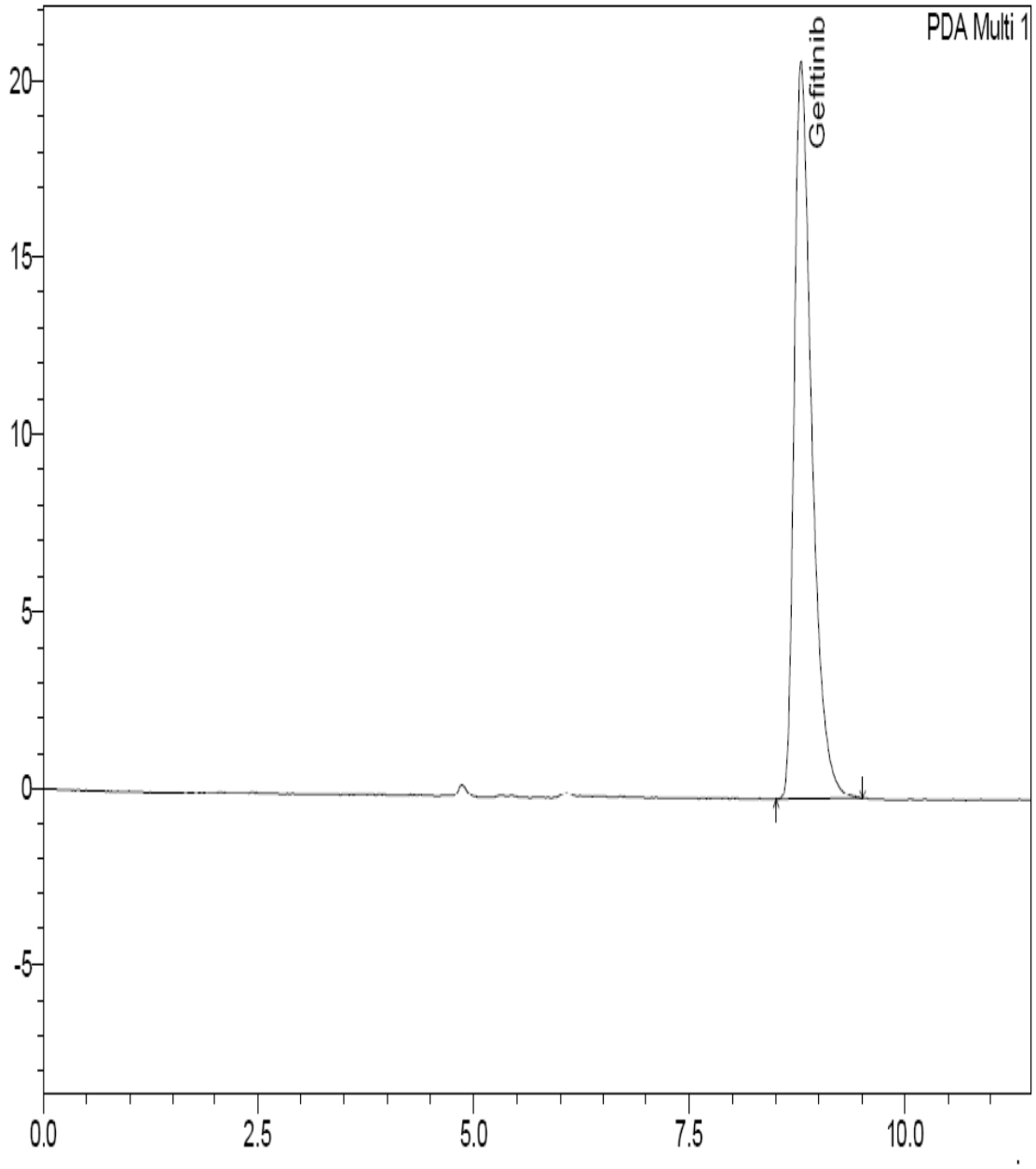


Fig : 4 – Chromatogram of Standard Gefitinib ***10µg/ml***

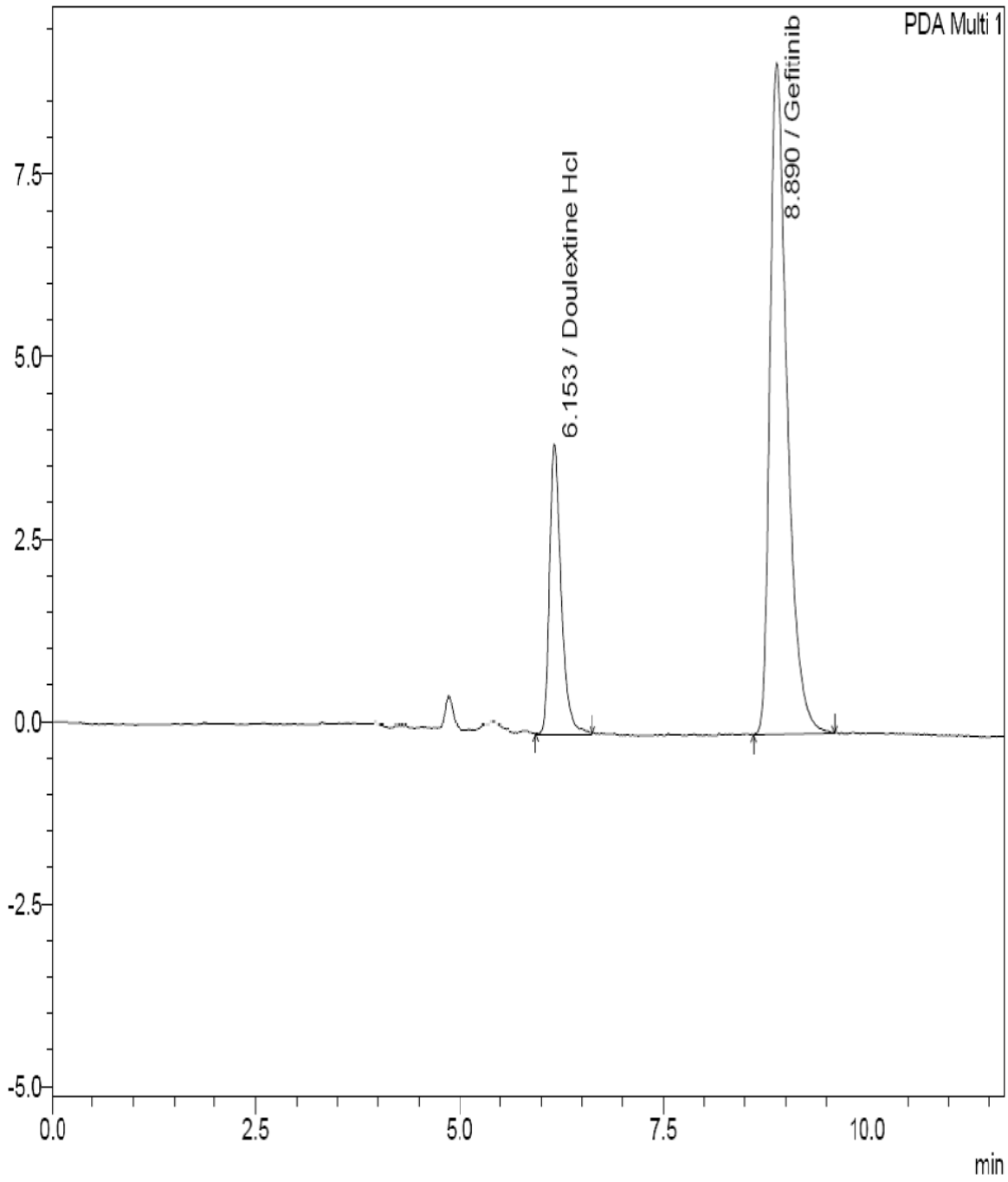


Fig *: 5 – Chromatogram of I.S and Standard Gefitinib* ***10µg/ml***

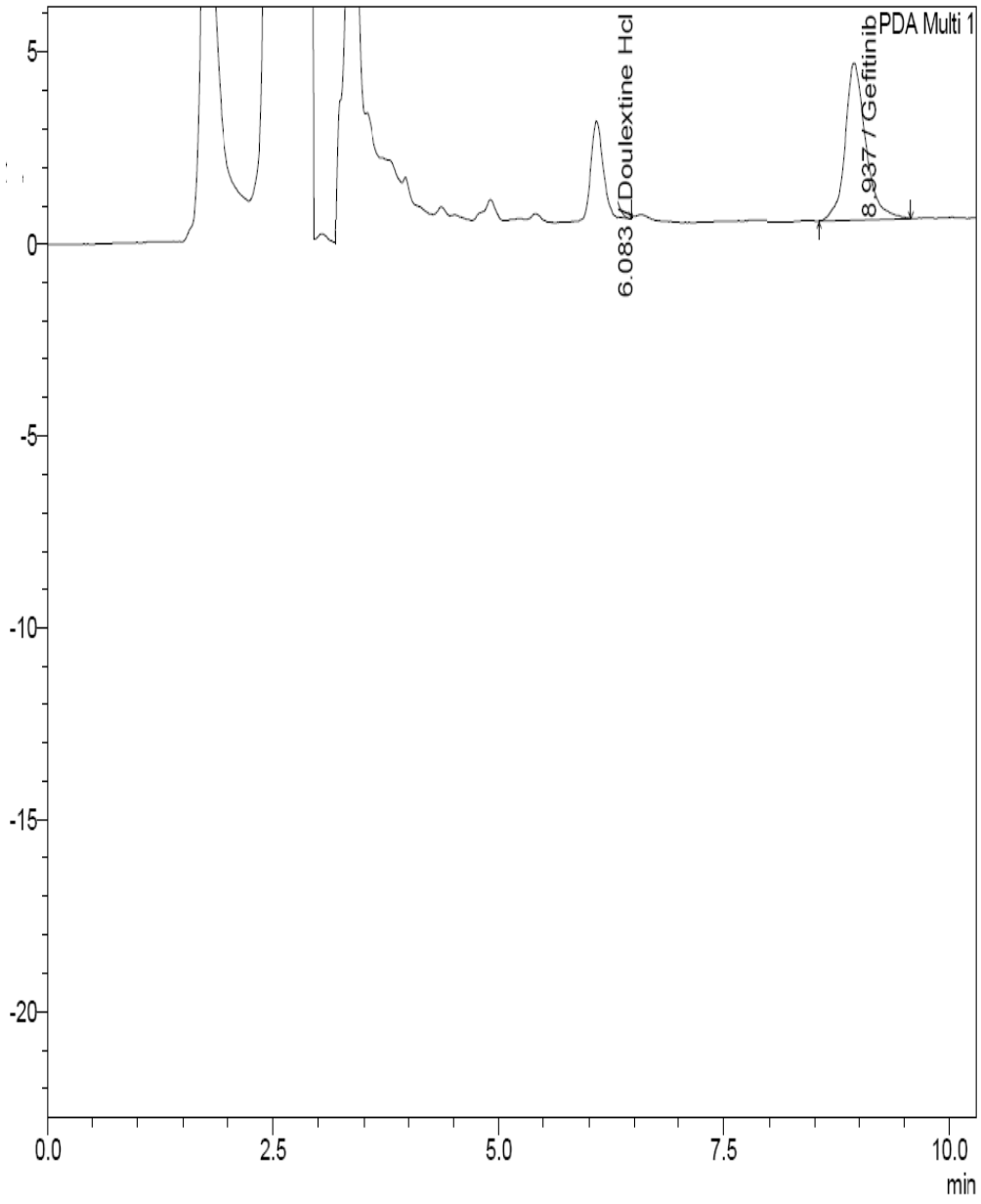


Fig : 6 - Chromatogram of I.S and Gefitinib in human plasma ***100ng/ml***

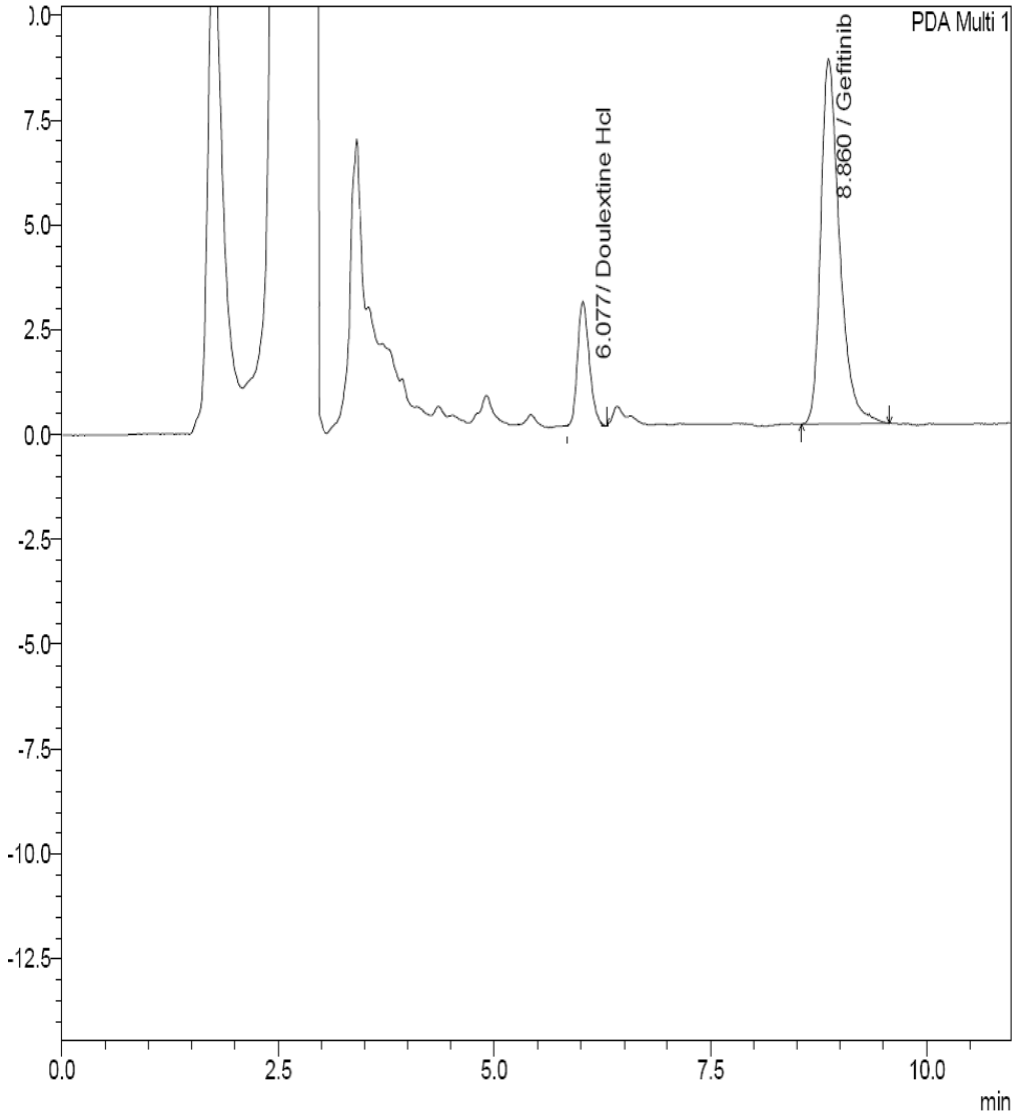


Fig : 7 - Chromatogram of I.S and Gefitinib in human plasma ***200ng/ml***

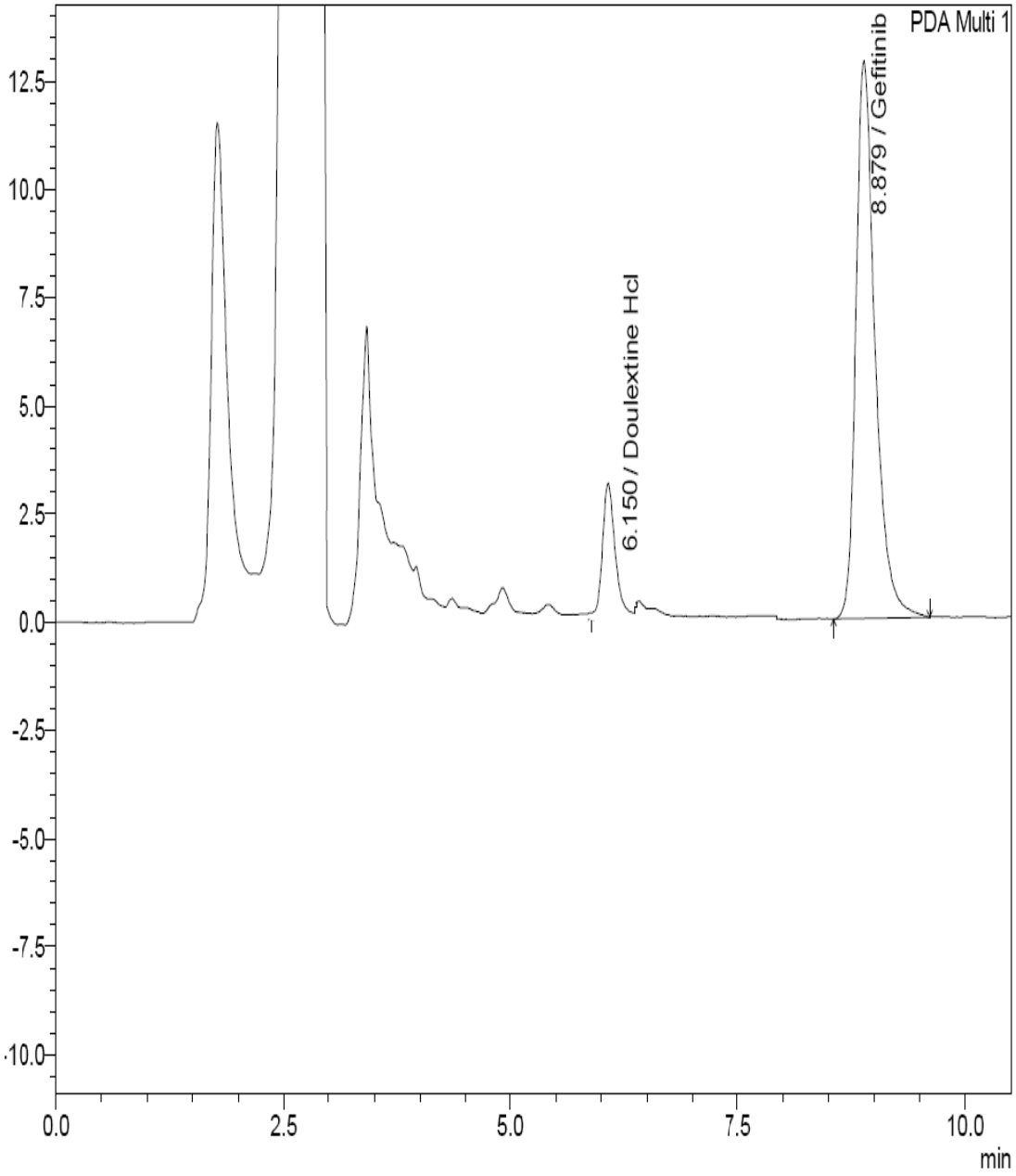


Fig : 8 - Chromatogram of I.S and Gefitinib in human plasma ***300ng/ml***

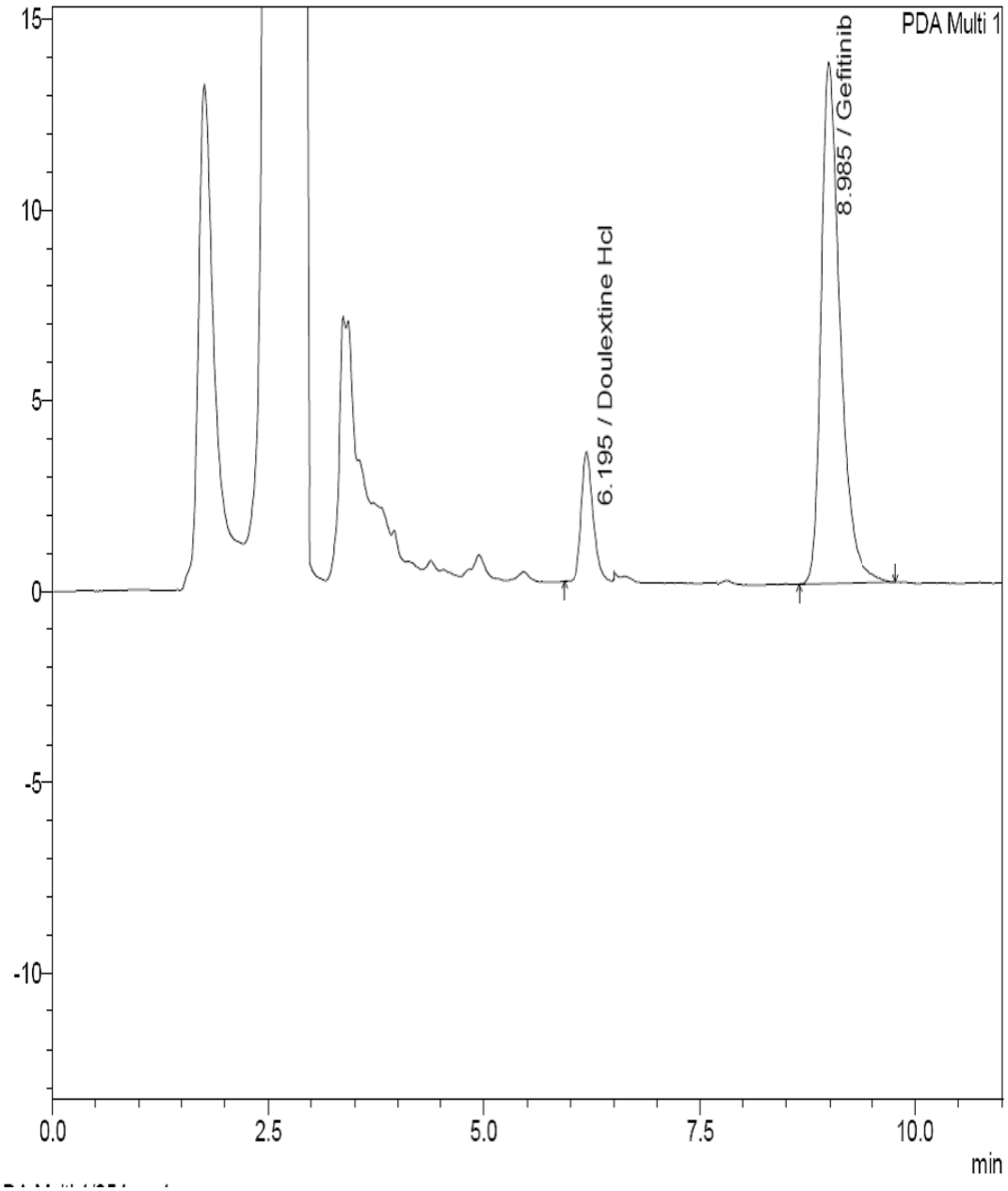


Fig : 9 - Chromatogram of I.S and Gefitinib in human plasma ***400ng/ml***

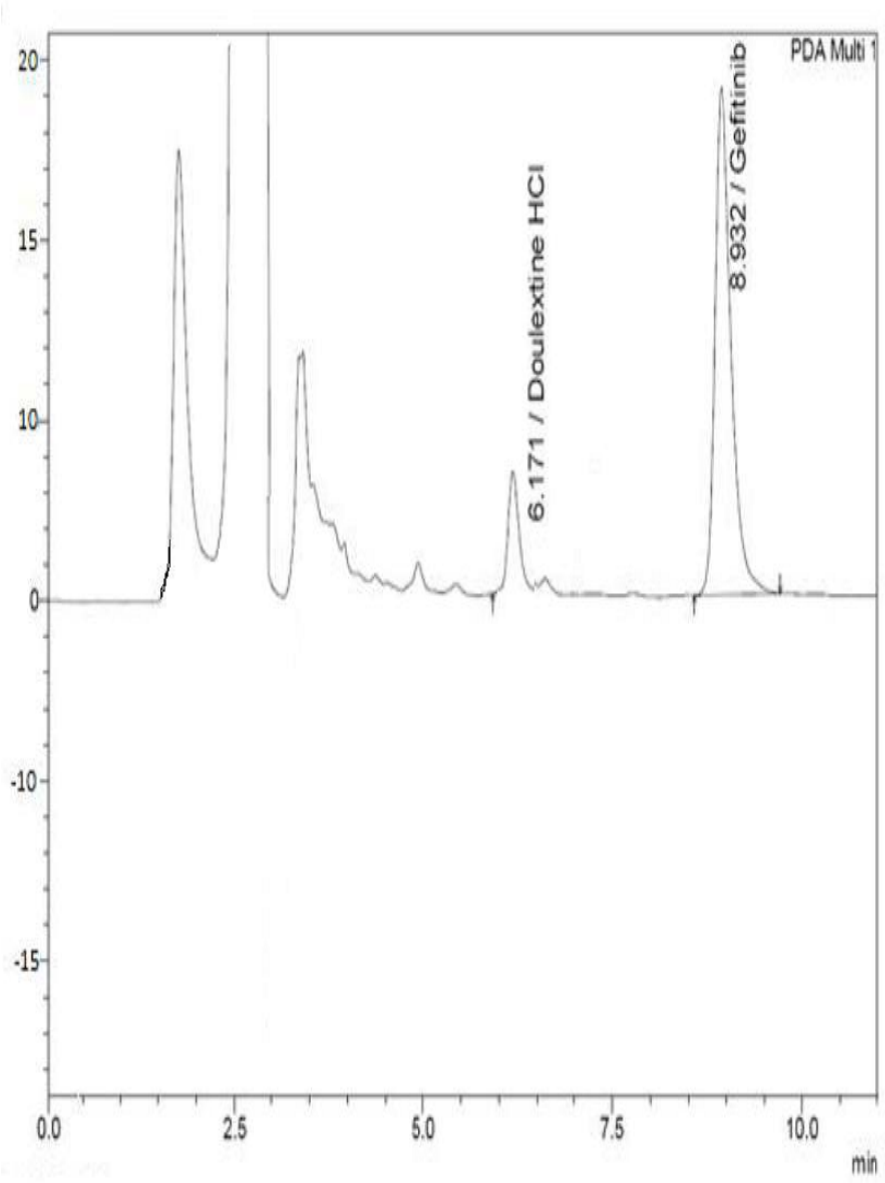


Fig : 10 - Chromatogram of I.S and Gefitinib in human plasma ***500ng/ml***

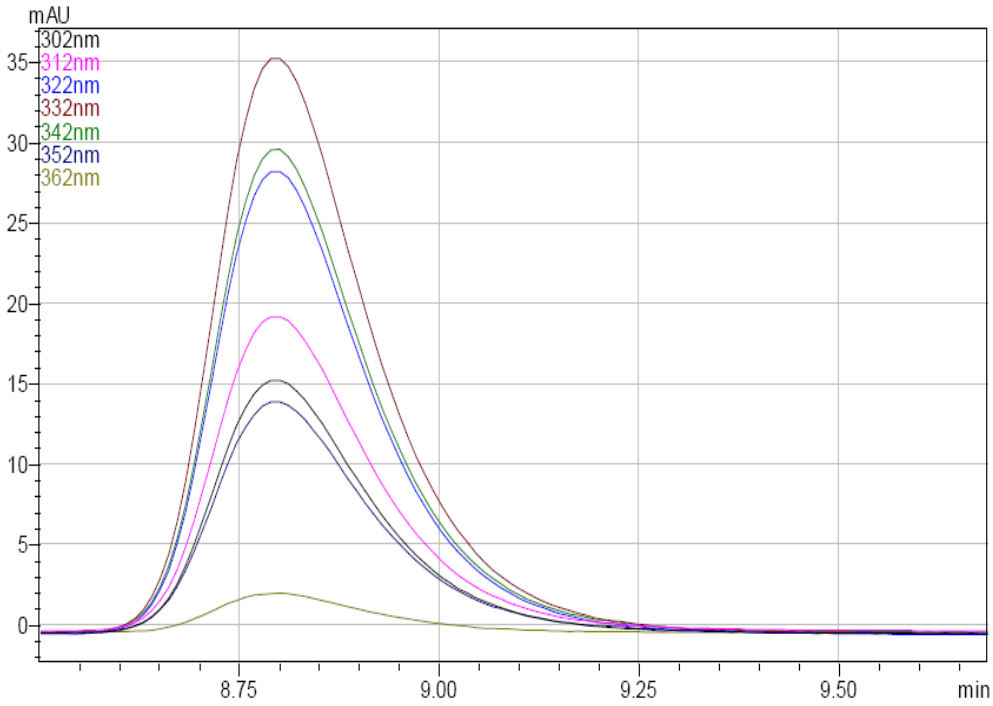


Fig : 11 - Peak profile of Standard Gefitinib

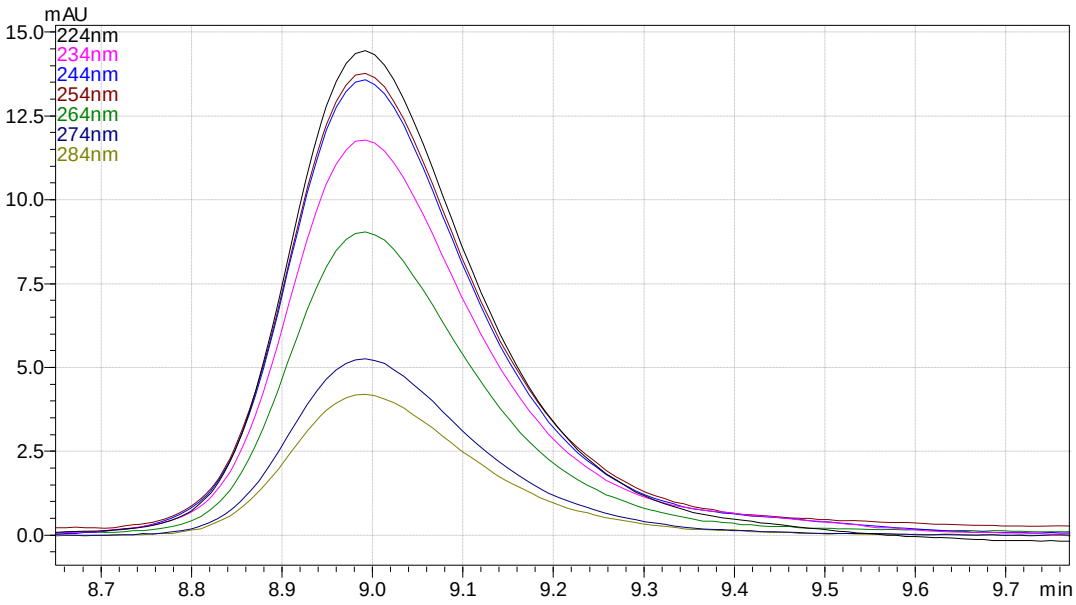


Fig : 12 – Peak profile of Gefitinib in Human plasma

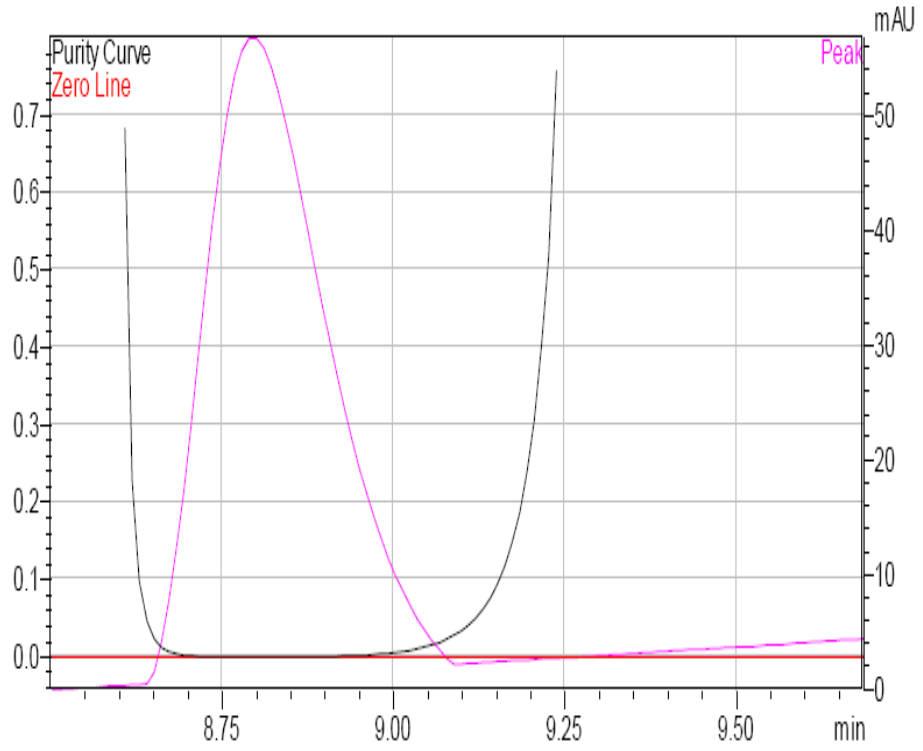


Fig : 13 – Peak purity of Standard Gefitinib

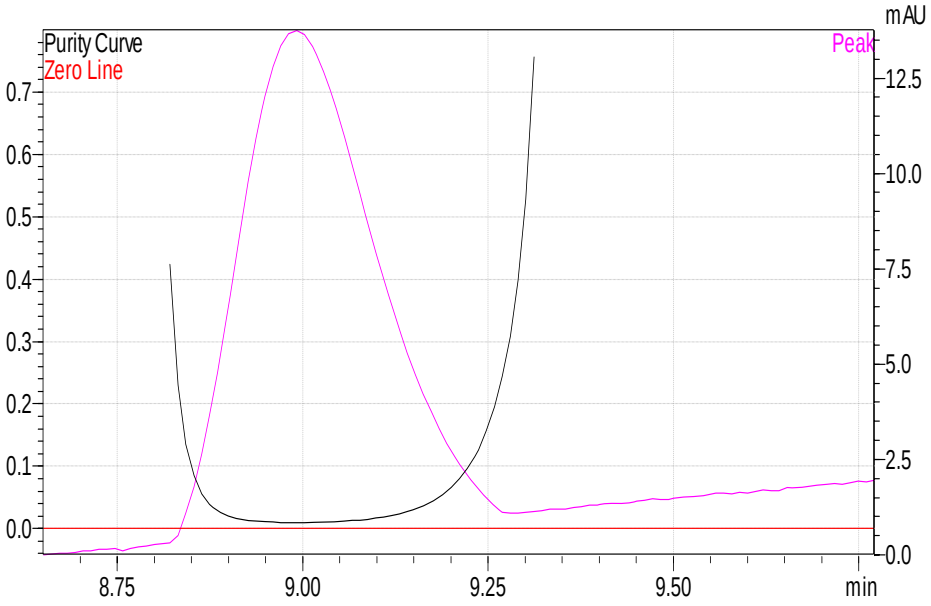


Fig : 14 – Peak purity of Gefitinib in Human plasma

CHAPTER -7

RESULTS & DISCUSSION

RESULTS AND DISCUSSION

1). FIRST DERIVATIVE SPECTROSCOPY METHOD

Estimation:

A First Derivative Spectroscopy method was developed for the estimation of Gefitinib in tablet dosage form, which can be conveniently employed for routine quality control in pharmaceutical dosage forms.

Gefitinib is an UV absorbing molecule with specific chromophores in their structure and it absorbs at a particular wavelength and this fact has been successfully employed for its quantitative determination by UV Spectrophotometric method.

The stock solutions and working standards are made in organic media (Methanol). The λ-max of the drug for analysis was determined by taking scan of the drug sample solution in the entire UV region (200–400 nm). All the method validation parameters are well within the limits.

In this method, 10 µg/ml working standard solution was prepared by appropriate dilution of standard stock solution in methanol and methanol was used as blank solution. This solution was scanned in the spectrum mode from 400 nm to 200 nm wavelength ranges and the first order derivative spectra were obtained at n=1, a sharp peak at 242 nm **(Fig : 2)**. A calibration curve was plotted by taking the absorbance difference (dA/dλ) against the concentration of standard stock solutions. By using the calibration curve, the concentration of the sample solution can be determined.

Table: 7. 1 **- Results of calibration curve at 242 nm for Gefitinib by First order derivative spectroscopy**

Sl. no.	Conc. (mcg / ml)	Absorbance at 242 nm
1	2	0.1771
2	4	0.334
3	6	0.504
4	8	0.6367
5	10	0.7895
6	12	0.9233

Table: 7. 2 - Optimum conditions, Optical characteristics and Statistical data of the Regression equation in First order Derivative Method

Parameter	First order Derivative Method
λ_{max} (nm)	242
Beer's law limits (µg / ml)	2-12
Molar extinction coefficient ($L.mol^{-1} cm^{-1}$)	$10.7415 X 10^5$
Sandell's sensitivity ($\mu g/cm^2$-0.001 absorbance units)	0.00416
Regression equation (Y*)	Y = 0.074x + 0.037
Slope (b)	0.074

Intercept (a)	0.037
Correlation coefficient (r^2)	0.998
Intraday Precision (% RSD**) Inter day Precision (% RSD**)	0.7712 0.7043
Limit of detection (µg / ml)	0.087
Limit of quantitation (µg / ml)	0.264

*Y= b C + a where C is the concentration of Gefitinib in µg/ ml and Y is the absorbance at the respective $\lambda_{max.}$

**Average of six determinations.

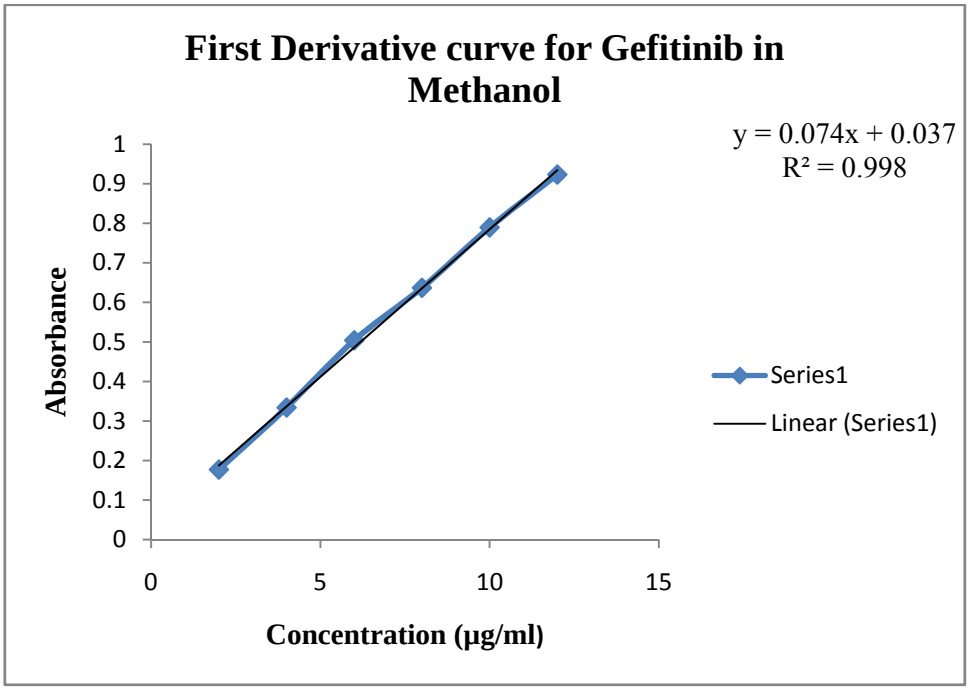


Fig : 15 – Calibration curve for Gefitinib in Methanol

Table: 7.3 - Determination of Precision for Gefitinib at 242 nm by First order derivative spectroscopy

Conc. mcg / ml	Intra-day Absorbance Mean ± SD**	% CV	Inter-day Absorbance Mean ± SD**	% CV
2	0.1771 ± 0.00196	1.1147	0.1791 ± 0.0021	1.1725
4	0.334 ± 0.001768	0.5285	0.340 ± 0.001778	0.5229
6	0.504 ± 0.00775	1.504	0.504 ± 0.00775	1.537
8	0.6367 ± 0.004932	0.7712	0.6417 ± 0.00452	0.7043
10	0.7895 ± 0.01007	1.2704	0.798 ± 0.01528	1.914
12	0.9233±0.00415	0.4489	0.9243±0.00515	0.5571

**Average of six determinations.

Table:7. 4 - Determination of Accuracy results for Gefitinib by First order derivative spectroscopy

Tablet	Amount of sample (µg / ml)	Amount of drug added (µg / ml)	Amount Recovered (µg / ml)	% Recovery ± SD**
Sample	10	5	14.48	99.33 ± 0.34
	10	10	19.92	99.21 ± 0.61
	10	15	25.58	100.87 ± 0.77

**Average of six determinations.

Table: 7.5 - Ruggedness results for Gefitinib at 242 nm by First order derivative spectroscopy

Tablet	Label claim (mg)	Analyst I		Analyst II	
		Amount found (mg)	Recovery ± SD** (%)	Amount found (mg)	Recovery ± SD** (%)
Sample	250	249.89	99.62 ± 0.08	249.984	99.45 ± 0.08

** Average of six determinations.

2). HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD

Bioanalytical method was developed for Gefitinib and it was validated for its transferability.

Chromatographic separation of standard Gefitinib:

The chromatogram of gefitinib was recorded alone and shown in figure **(Fig: 4).**The standard solution which contains internal standard Duolextine hydrochloride was injected with the developed chromatographic conditions, and the chromatograms were recorded and shown in figure **(Fig: 5).**

The retention time of Gefitinib and internal standard (Duolextine Hcl) was 6.1 and 8.8 min respectively with percent RSD of less than 2%. The results are shown in table **(Table 7.6)**. The peak purity study reveals that signal ratios (relative absorbance at different wavelengths) were constant across the peak profile of Gefitinib. The peaks obtained in the present study were symmetric, good and no interference was observed between the peaks.

The method developed was advantageous than the reported methods by its lesser precision values and increased accuracy values. The run time of 10 minutes makes the method rapid and economical than the previously reported methods.

Chromatographic separation of Gefitinib in biological fluid:

Chromatogram of the blank plasma was recorded at the fixed chromatographic conditions and shown in figure (**Fig: 3)**.

Various precipitating agents were used for extraction of gefitinib in human plasma. But, out of all precipitating agents acetonitrile and methanol mixture was proved to be good because of its maximum percentage recovery. Chromatograms of the precipitating agents used for extraction process of plasma were recorded and the percentage recoveries were calculated and shown in table **(Table: 7.7).** The retention time for Gefitinib and internal standard (Duolextine HCl) were 6.1 and 8.8 minutes respectively as shown in **(Fig: 5)**. The peaks were symmetric with straight baseline.

The extraction method used for the present study was simple and newer than previous methods. In most of the reported methods used for plasma for extraction of drug was protein precipitated with acetonitrile but, for present method acetonitrile –methanol mixture was selected because of its maximum recovery of drug from plasma and it is advantageous.

METHOD VALIDATION

a) Accuracy and precision:

At two –levels these accuracy and precision studies were conducted i.e. intra-day and inter-day. In this the present developed method, shown the good accuracy and precision. Accuracy ranges from 99.4% to 100.4% with the precision 5.82% to 6.94% in intra-day method. In inter-day method the accuracy ranges from 99.7% to 101.07% with the precision 6.65% to 7.88%. Finally the data obtained here, was found to be within limits as per ICH guidelines and method was accurate.

Intra-day studies: In this plasma concentration 100-500 ng/ml were injected six times and mean peak area was calculated separately for each concentration and from that accuracy and precision percentage RSD values were calculated and shown in table **(Table: 7.8)**

Inter-day studies: In this the plasma concentrations of 100-500 ng/ml were injected into HPLC six times in three different days and mean peak areas were calculated and from that accuracy and precision percentage RSD were calculated and shown in table **(Table: 7.9).** The percentage relative standard deviation of precision for gefitinib was less than 15% for the bioanalytical study. The results obtained were within limits.

Acceptance criteria: *The percentage RSD value should be less than 15% for bioanalytical study.*

b) Linearity and range:

This method proved to be linear between ng/ml of gefitinib in human plasma, with a typical calibration curve of correlation equation y =50.49. x + 19.42, correlation coefficient > 0.999 shown in table **(Table: 7.10).**

The chromatograms of the plasma calibration standards with concentrations 100, 200, 300, 400 and 500 ng/ml were recorded and shown in figures **(Fig: 6, 7, 8, 9 and 10)** and their peak areas of both drug and internal standard were noted. The calibration curve for Gefitinib was plotted as peak response Vs concentration of the gefitinib calibration standards in plasma was shown **(in Fig: 16).** As we were using internal standard peak response was calculated for calibration curve. Peak response is the ratio of internal standard peak area to drug peak area. The correlation coefficient of Gefitinib shown was 0.999 which was within limits. This calibration curve plotted was linear and showed that the method had adequate sensitivity to the concentration (100 ng/ml-500 ng/ml) of the drug. Finally the data obtained, in this was within limits. Coefficient of correlation of Gefitinib was found to be less than 0.99.

Acceptance criteria: *The correlation coefficient should not less than 0.99*

c) Lower Limit of Quantification:

The LLOQ is the smallest concentration of the analyte, which shows response that can be accurately quantified and LLOQ = 10 × D/S, where, D is the standard deviation of y – intercepts of regression line and S is the slope of the calibration curve. This signal to noise ratio were performed by comparing measured signal of known low concentration of drug with those of blank plasma sample.

The Lower limit of quantification (LLOQ) for Gefitinib were separately determined and reported, based on the calibration curve for spiked plasma solutions was found to be 8.5 ng/ml respectively.

d) Recovery from plasma:

A recovery study for Gefitinib in plasma using acetonitrile-methanol mixture was shown in table **(Table: 7.11)**. With concentrations 100 ng/ml, 200 ng/ml, 300 ng/ml of Gefitinib recovery was calculated and showed 100.2%, 99.4%, 100.4% relative recoveries and percentage RSD as 8.6%, 7.9% and 7.3% respectively. From the data

Obtained, it was observed that the recovery of drugs in plasma was found to be 9% which is sufficient for bio-analytical study.

Acceptance criteria: *For an assay method, mean recovery should be 85-105% ± 2%.*

e) Ruggedness:

It expresses the precision within laboratories variations like different days, different analyst, and different equipments. Ruggedness of the method was assessed by spiking the plasma standard 6 times in two different days with different analyst and the standard solutions were analyzed by a different chemist and same instruments on a different day had been performed the reports were shown in table **(Table: 7.12)**.

The deviation among the results obtained by two chemists on a different day was well within the limits. Hence the method was rugged.

Acceptance criteria: *The percentage RSD should be less than 15%.*

f) Specificity:

For specificity the peak purity studies were done. Here for Gefitinib the peak purity index was 000 and the peak properties like peak profile were good for both standard and the sample. The peak purity and peak profiles for Gefitinib standard and sample were shown in figures **(Fig: 11, 12, 13 and 14)** respectively. By the data obtained in this, the present method developed was specific as values were within limits.

Acceptance criteria: *Purity angle should be less than purity threshold i.e.0.99-1.00*

g) System suitability:

The parameters such as column efficiency (theoretical plates), resolution factor and peak asymmetry factor, HETP, tailing factor, LLOQ of the optimized methods was found satisfactory. The results of the system suitability studies in plasma were shown in table **(Table: 7.13)** these parameters were shown to be within specified limits.

Table:7. 6 - Retention time of Gefitinib and Duolextine HCl (IS)

S. No	Method	Retention time of Drugs (min)	
		Gefitinib	Internal Standard (Duolextine HCl)
1	HPLC	8.893	6.122
2		8.910	6.171
3		8.860	6.195
4		8.937	6.077
5		8.879	6.150

Table: 7.7 - Recovery study of Gefitinib:

Level	Conc. of drug added (ng/ml)	Amt. of drug recovered from plasma (ng/ml)			% Recovery		
		ACN	Methanol	ACN and methanol mixture	ACN	Methanol	ACN and methanol mixture
I	50	42.4	32.4	48.2	84.8	64.8	96.4
II	100	88.2	74.6	97.2	88.2	74.6	97.2
III	150	143.9	147.4	147.4	95.9	80.9	98.2

Table: 7.8 - Accuracy and Precision Studies of Gefitinib (Intraday)

Sl.No	Conc. of Drug (ng/ml)	Mean Peak Area	Accuracy (%)	RSD (%)
1	100	5080	100.2	5.82
2	200	10060	99.4	6.94
3	300	15228	100.4	6.45

**Average of six determinations.

Table: 7.9 - Accuracy and Precision Studies of Gefitinib (Interday)

Sl.No	Conc. of Drug (ng/ml)	Mean Peak Area	Accuracy (%)	RSD (%)
1	100	5190	101.07	7.88
2	200	10170	99.7	6.65
3	300	15338	100.4	6.85

**Average of six determinations.

Table: 7.10 - Calibration Standards Peak Area

Concentration (ng/ml)	Peak Area
0	0
100	5080
200	10060
300	15228
400	20320
500	25167

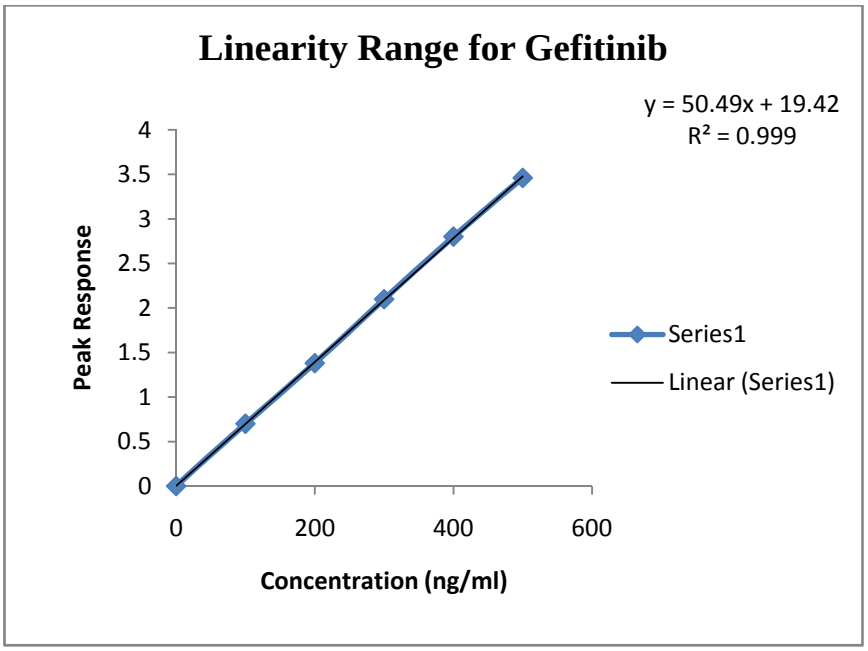


Fig : 16 – Calibration curve for Gefitinib

Table: 7.11 - Recovery studies of Gefitinib

Levels	Conc. of Drug added (ng/ml)	Amount of drug recovered in plasma sample (ng /ml)	Relative Recovery (%)	% RSD
I	100	100.2	100.2	8.6
II	200	198.8	99.4	7.9
III	300	301.2	100.4	7.3

**Average of six determinations.

Table: 7.12 - Ruggedness Studies for Gefitinib

Drug	Concentration (ng/ml)	Mean Peak Area	%RSD
	Day I Analyst – I		
Gefitinib	100	5080	6.44
	Day II Analyst - II		
Gefitinib	100	5291	7.78

**Average of six determinations.

System suitability:

Column efficiency (theoretical plates), resolution factor and peak asymmetry factor, HETP, tailing factor, LLOQ are the system suitability parameters. These parameters of the optimized methods were found satisfactory. The results of the system suitability studies in plasma were shown in table **(Table: 7.13).** These parameters were shown to be within specified limits.

Table: 7.13 - System suitability studies

Sl.No	Parameters	Gefitinib
1.	Theoretical Plate	7391.60
2.	Tailing Factor	0.96
3.	HETP	20.293
4.	LLOQ	8.5 ng/ml
5.	Resolution	4.14
6.	K	4.0718

CHAPTER -8

SUMMARY & CONCLUSION

SUMMARY AND CONCLUSION

HPLC METHOD

A bioanalytical method was developed for the estimation of Gefitinib by HPLC method. The method was validated for its transferability to other user or other laboratory. The HPLC method developed uses Phosphate buffer + TEA with pH 6.5 adjusted with Orthophosphoric acid and acetonitrile in meticulous ratio. The peaks obtained for the drug of interest and internal standard by the present method are well resolved from each other without any interference and from the plasma endogenous proteins. The peaks are symmetrical with acceptable tailing factor. The retention time of Gefitinib and that of internal standard was shorter and proves the method is rapid.

The results of linearity, intraday and interday precision study and capability of the extraction method were within the limits of bioanalytical method development. The method was linear with a correlation coefficient of acceptable agreement, which is suitable for the estimation of Gefitinib in plasma and other biological fluids.

The method demonstrated relative recoveries with acceptable relative standard deviation. The Lower limit of quantification (LLOQ) for Gefitinib was found to be nanograms lesser than unity. Hence the developed method is sensitive for the estimation of Gefitinib in trace amounts.

Peak purity studies, with peak purity index values closer to unity reveals that the method developed was specific for the estimation of Gefitinib in blood and other biological fluids.

FIRST DERIVATIVE SPECTROSCOPIC METHOD

The objective of the present work also expands to develop a new simple First Derivative Spectroscopic method for the estimation of Gefitinib in tablet dosage forms.

From the extensive literature survey for analytical method, it was observed that only less instrumental methods have been reported to determine Gefitinib in formulation.

Since the selected formulation is a single component system, an attempt was made to develop a First derivative Spectroscopic method for the estimation of Gefitinib in tablet dosage form.

The method is based on the absorbance of chromophores of Gefitinib in the UV region. Methanol was selected as a solvent, because in this solvent the drug gives good absorbance in the UV region.

The linearity and range was established and it was found to be in the range of 2 to 12 μg/ml for the drug. The correlation coefficient of Gefitinib at 242 nm was found to be 0.998. The method was validated for accuracy and precision. The percentage recovery of Gefitinib was found to be 99.33 % from the formulation.

The good percentage recovery clearly confirms the reproducibility and accuracy of the developed method. Similarly the %RSD value for precision was also within the acceptable limits.

The developed First Derivative Spectroscopic method has the following advantages:

- The standard and sample preparation requires less time
- No tedious extraction procedure were involved
- Suitable for the analysis of raw materials

Conclusion

From the current work it was finally concluded that the developed RP-HPLC method in human plasma and First derivative spectroscopic method were found to be very simple, reliable and selective for providing satisfactory accuracy and precision. The methods are suitable for routine quantitative analysis in pharmaceutical dosage forms.

In this present study the retention time of Gefitinib was found to be 8.8 min respectively. Furthermore the method has been shown to be specific and selective.

The method developed can be used in

- Therapeutic drug monitoring units.
- Bioequivalence and bioavailability studies of Gefitinib.
- Pharmacokinetic and bio-equivalence study centers.
- Toxicology study of Gefitinib.

CHAPTER -9

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